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DEUTERIUM ISOTOPE EFFECTS IN CHEMISTRY AND BIOLOGY*

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DEUTERIUM IN BIOLOGY

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Deuterium was discovered in 1932 by Urey *et al.*,[1] who found that the amount of deuterium in protium is about 1 part in 4000.[2] The simplest deuterium-containing compound, heavy water, differs from ordinary water in many physical properties: it has a higher density and viscosity, higher melting and boiling points, and is a poorer solvent for salts and gases.[3] Reaction rates involving the rupture of deuterium bonds are also different from those involving hydrogen. Calculations from absolute rate theory indicate that the carbon-hydrogen bond may be broken 7 times faster than the carbon-deuterium bond, the N—H bond 8.5 times faster than N—D, and the O—H bond 10.6 times faster than O—D.[4] These differences in properties might be expected to give rise to differences in the chemical and biological activities of deuterium-containing compounds.

Urey[5] and G. N. Lewis[6] recognized the biological implications of these differences. Experiments carried out one quarter of a century ago pointed to differences in the growth of tumors[7] and tissue cultures of various cells[8] under the influence of deuterium oxide. Some enzyme systems were also studied in D_2O.[9] A number of observations exist relating to the variable toxicity of deuterium oxide in diverse biological systems.[10]

Today, with the relative availability of deuterium, the great advances in our knowledge of biochemical and physiological mechanisms, and newer and more refined techniques, we are in a position to elucidate the mechanisms by which deuterium exerts its effects. Active research in this field is under way at a number of institutions, and it is our hope that this monograph will serve as a means of bringing this exciting tool to the attention of a wider segment of the scientific community.

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STEREOCHEMICAL AND KINETIC APPLICATIONS OF DEUTERIUM ISOTOPE EFFECTS

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Substitution of deuterium for hydrogen causes a number of observable effects of various kinds that can be very useful as theoretical tools. These effects are generally understood in terms of the effect of mass on vibrational energy levels. For most bonds the first vibrational level alone is importantly populated at ordinary temperatures. To the extent that such vibrations may be considered as harmonic oscillators the energy of this level is given by

$$E_0 = \frac{1}{2} h\nu = \frac{1}{2} hc \sqrt{\frac{k}{\mu}} \tag{1}$$

in which h is Planck's constant, c is the velocity of light, k is a force constant and μ is the reduced mass that approximates 1 and 2, respectively, for bonds to hydrogen and to deuterium.[1] The energy of the first vibrational level, the zero point energy, is lower for a deuterium-containing bond. One result is that excitations to higher vibrational levels require lower energy for vibrations of deuterium-containing bonds; hence, the infrared spectra of deuterium compounds usually show dramatic changes from the corresponding hydrogen compounds; shifts in some bands occur to longer wave lengths. This effect is often useful not only in analyses but in determining the structures of deuterated compounds.

If the transition state for a reaction involves a decrease in the force constant of a bond, the zero point energy is also decreased. Compared to a bond to hydrogen, a deuterium-containing bond has less zero point energy to begin with and loses less in going to the transition state. The activation energy is greater and the rate of the reaction is correspondingly less for the deuterated compound. This effect is illustrated in FIGURE 1.

If the reaction involves transfer of the hydrogen to a new atom, the resulting primary isotope effect can have rather substantial magnitude. In a recent study Saunders and Edison[2] found a primary deuterium isotope effect, k_H/k_D, of 7.9 at 30° C. for the reaction

$$(CH_3)_3COK + C_6H_5CD_2CH_2Br \xrightarrow{t\text{-BuOH}} C_6H_5CD{=}CH_2 + (CH_3)_3COD + KBr$$

in which a hydrogen or deuterium is removed in the rate-determining step.

Smaller but still important kinetic effects are often also found in cases in which the hydrogen or deuterium is not directly involved in the reaction. One such effect that we may call a secondary isotope effect of the first kind results from a change in hybridization of a C—H or C—D bond in going to the transition state. Such a change in hybridization is often accompanied by a change in force constant for a vibration with a consequent isotope effect on the rate.

An early example was found in the solvolysis of cyclopentyl-1-*d* *p*-toluenesulfonate in acetic acid, for which $k_H/k_D = 1.15$ at 50° C.[3,4] This reaction involves an intermediate of the carbonium ion type. The experimental effect has been shown to be interpretable as a change from a tetrahedral C—H bending vibration of the ground state to an out-of-plane bending motion of the transition state, which has the character of a nearly planar trigonal carbonium ion. As illustrated in FIGURE 2, this vibration is freer in the trigonal compound; thus it occurs at lower frequencies and has a lower zero-point energy. The heavier deuterium atom benefits less from this lower vibration; hence, the deuterium compound solvolyzes at a slower rate. This effect has been amply confirmed in both magnitude and direction.[5-10] One further point, however, is of interest. The experimental isotope effect is of smaller magnitude than that calculated from the theoretical model, ~1.3.[4] This difference has been explained in part as resulting from the proximity of the leaving p-toluenesulfonate anion in the transition state, which would be expected to impede the vibration somewhat. Johnson and Lewis[7] have shown that the magnitude of the isotope effect correlates rather well with relative proximities of entering and leaving groups in various displacement reactions; hence, this isotope effect may prove to be an important tool in determining the geometries of transition states.

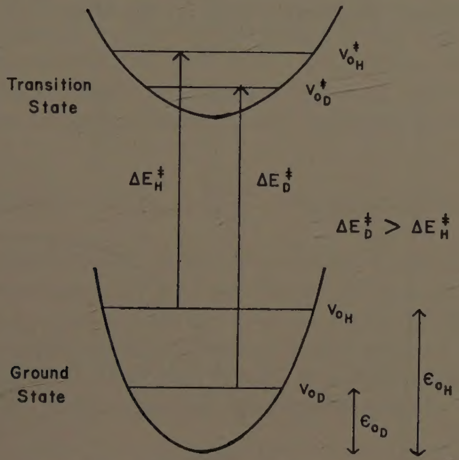


FIGURE 1. An illustration of the effect of zero-point energy differences on reaction rate. Reproduced by permission from *Chemical Reviews*, **56**: 672 (1956).

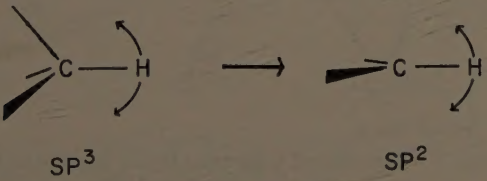


FIGURE 2. The change in frequency of the bending vibration gives rise to an isotope effect.

An obvious corollary of this effect is that the change from a trigonal to a tetrahedral configuration should be accelerated by an α-deuterium atom. This corollary has been confirmed in some reactions of double bonds.[9,10] The relative magnitudes of the effects may be useful in determining the geometries of these transition states as well. It should be mentioned, however, that these interpretations may be complicated by an electronic effect of α-deuteriums that is discussed below.

Other effects of substitution of hydrogen by deuterium are not readily interpretable as changes in force constants. These additional effects result because bonds are not harmonic oscillators; that is, the effects are said to be due to anharmonicity. The potential well is not parabolic but is skewed. A consequence, which is exaggerated for emphasis in FIGURE 3, is that a deuterium atom is closer on the average to the bonded atom than is a corresponding hydrogen.

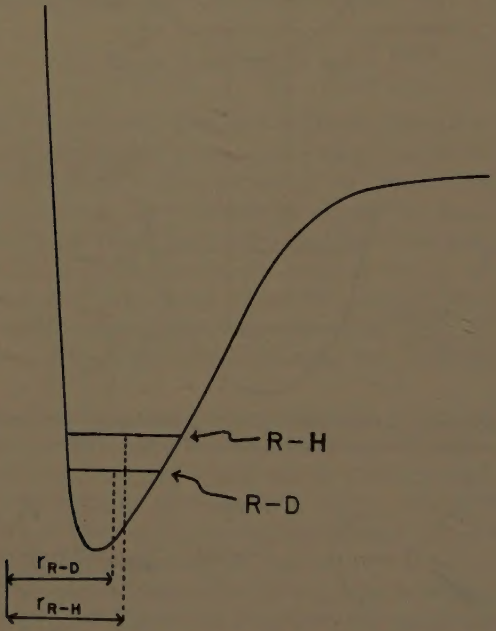
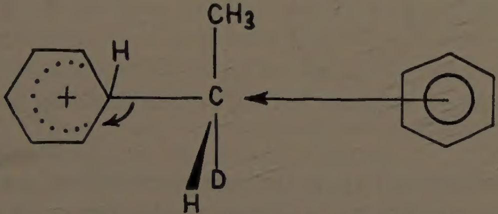


FIGURE 3. The effect of anharmonicity on mean bond length.

A slightly shorter C—D bond distance, on the average, further implies a slightly higher electron density about carbon and a slightly different optical polarizability along the bond than for a C—H bond. A corollary of this latter difference is that compounds of the type, R^1-CHD-R^2, should possess optical activity. Such optical activity in hydrocarbons was demonstrated by Alexander and Pinkus[11,12] and by Eliel.[13] Eliel prepared ethylbenzene-α-*d* having $[\alpha]_D$ −0.30° C. by the reaction of optically active α-phenethyl chloride with lithium aluminum deuteride. Subsequent work showed that Eliel's hydrocarbon was not optically pure; the rotation of optically pure ethylbenzene-α-*d* is $[\alpha]_D$ −0.7° C. Hydrogen-deuterium asymmetry clearly can confer sufficient optical rotatory power to be useful in stereochemical studies. Shortly after the preparations of these hydrocarbons, optically active butanol-1-*d* was prepared as the first synthetic example having a primary asymmetric carbon that carries a functional group.[14] This alcohol was prepared by a partially asymmetric reduction and was not optically pure. At about the same time, however, a single enantiomer of ethanol-1-*d* was prepared by Loewus *et al.*,[15] who used an enzymatic reduction. These compounds and their simple functional derivatives have proved to be very useful in studies of the stereochemistry of reactions at primary centers.[14-24] The unique power of this tool is exemplified in a recent study of the stereochemistry of disproportionation of ethylbenzene. Optically active ethylbenzene-α-*d* labeled in the ring with C^{14} was allowed to react with a solution of gallium bromide and hydrogen bromide in benzene at 50° C.:

$$^*C_6H_5CHDCH_3 + C_6H_6 \xrightarrow[HBr]{GaBr_3} {}^*C_6H_6 + C_6H_5CHDCH_3$$

The rates of racemization and of loss of radioactivity from the ethylbenzene were determined simultaneously and were found to be equal. If the exchange of benzene rings had occurred with inversion of configuration, the rate of racemization would have been double the rate of loss of radioactivity. The experimental result, found by L. Reif and myself in unpublished work, rules out a displacement reaction mechanism for disproportionation:



Indeed, further observations of the same system have led to a completely new mechanism for the disproportionation reaction.

The anharmonicity explanation for the optical activity of compounds with hydrogen-deuterium asymmetry implies that such compounds should show optical dispersion curves similar to those of "normally" optically-active compounds. V. Sarich and I, in unpublished work, have examined the dispersion curves for ethanol-1-*d* and butanol-1-*d*, among others, and found a close similarity to that of 2-octanol considered on the same scale.

The slightly greater electron density near carbon in a C—D bond, compared to a C—H bond, because of anharmonicity means that effectively deuterium should have an electron-donating inductive effect. This effect has measurable magnitude and is manifested physically in different dipole moments[25] for HCl and DCl and for NH_3 and ND_3, and by different nuclear quadrupole coupling constants and NMR chemical shifts in alkyl halides.[26-30]

Halevi and Nussim[31-33] have reported that deuterium reduces acidity; $C_6H_5CD_2COOH$, CD_3COOH, and $C_6H_5CD_2NH_3^+$ are 10 to 13 per cent weaker acids than are the corresponding undeuterated compounds. In agreement with these observations, Lewis and Farrissey[34] found that $(C_2H_5)_2NCD_2CH_3$ is 7 per cent more reactive than triethylamine toward methyl *p*-bromobenzenesulfonate. Similarly, Elliott and Mason[35] have recently reported that ring-deuterated aniline reacts faster than aniline with benzoyl chloride. These effects of α-deuterium substitution that behave as inductive effects may be called secondary isotope effects of the second kind.

A secondary kinetic isotope effect caused by β-deuterium substitution may be mentioned at this point. Shiner[36] compared the solvolysis of $(CD_3)_2CCl\text{-}CD_2CH_3$ in 80 per cent aqueous ethanol with that of *t*-amyl chloride and found $k_H/k_D = 2.36$. A number of further examples have confirmed that the rate of formation of carbonium ion intermediates is reduced by the presence of β-deuteriums.[4,37-43] There seems to be general agreement that the effect is due to an inhibition of hyperconjugation by the deuterium[4,44,45] although there has been some controversy over the mechanism of this inhibition. Lewis and Boozer[38] and Shiner[36] have suggested that hyperconjugation causes a decrease in force constants of C—H bonds in carbonium ions. My associates and I have suggested that a decrease in the force constant of a specific bending mode is principally responsible.[4] Halevi and Nussim,[46] however, suggest that the increased amplitude of C—H bending vibrations is responsible for the isotope effect. Their view is illustrated in FIGURE 4. Because of this increased amplitude, which is augmented by anharmonicity in the bending vibrations of the C—H bonds of the methyl group, the hydrogens are spread farther apart, on the average, than are the deuteriums, and overlap better with the vacant p-orbital of the carbonium ion results. The controversy is not settled yet, although current evidence would seem to favor the Halevi-Nussim view.

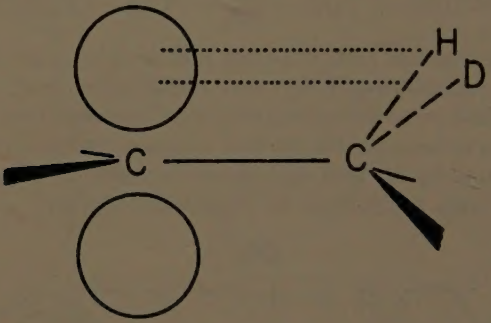


FIGURE 4. The greater amplitude of C—H bending vibrations results in better hyperconjugation, on the average.

A number of these isotope effects have been used jointly to gain an understanding of some base-catalyzed exchange reactions of hydrocarbons. In the presence of lithium cyclohexylamide in cyclohexylamine solution, toluene-*α-d* loses its deuterium with a half-life of a few hours at 50° C., (as determined by

D. E. Van Sickle and myself in unpublished experiments). Careful vacuum line techniques resulted in good reproducible pseudo-first order kinetics; the loss of deuterium was followed by quantitative infrared analyses. The isotope effects were used to test the hypothesis that this reaction proceeds through electron-rich intermediates of the benzyl anion type.

A mixture of $C_6H_5CHDCD_3$ and $C_6H_5CHDCH_3$ was allowed to react under these conditions and samples of the hydrocarbon were isolated at intervals and analyzed for the various deuterated species by mass spectrography.* The data were readily interpreted to yield $k_H/k_D = 1.10 \pm .02$; that is, the tetradeutero compound lost its α-deuterium at a 10 per cent slower rate than did the monodeuterohydrocarbon. This result resembles those summarized above for the effect of deuterium on acid strength, and implies that the central carbon atom bears a substantial negative charge at the transition state.

A similar study with a mixture of toluene-α-d and toluene-α,α,α-d_3 showed that $k_1/k_3 = 1.30 \pm .02$, for rate constants defined by the reactions:

$$C_6H_5CD_3 \xrightarrow{3k_3} C_6H_5CD_2H$$

$$C_6H_5CH_2D \xrightarrow{k_1} C_6H_5CH_3$$

This isotope effect has the magnitude and direction expected for a transition to a trigonal carbanion configuration, although this explanation is complicated by an "inductive" effect of deuterium of unknown magnitude.

W. C. Langworthy and myself, in unpublished work, obtained the primary isotope effect by determining simultaneously the loss of deuterium from toluene-α-d and the loss of tritium from toluene-α-t. Two runs gave $k_D/k_T = 2.75$ and 2.98. Using a treatment derived from Swain *et al.*,[47] these figures may be converted to the corresponding values for k_H/k_D, 9.9 and 11.8 respectively. A similar reaction with ethylbenzene-α-d and ethylbenzene-α-t gave $k_D/k_T = 3.0$ and $k_H/k_D = 12$.[48] These large primary isotope effects clearly mean that the C—D bond is largely broken at the transition state.

A combination of these effects was used to study the stereochemistry of the reaction with optically-active ethylbenzene-α-d. Racemization of this hydrocarbon under the reaction conditions can occur from replacement of deuterium by hydrogen (k_D) and in some fraction, λ, of the replacements of the α-hydrogen by hydrogen (k_H):

$$k_{rac} = k_D + \lambda k_H$$

Determination of λ characterizes the stereochemistry of the reaction; $\lambda = 0$, 1 or 2 for complete retention, racemization, or inversion, respectively. Using the primary isotope effect above, k_H is evaluated from k_D. A simultaneous determination of k_{rac} and k_D showed that this reaction proceeds with 17 per cent racemization and 83 per cent net inversion of configuration.[48] The application to this reaction of isotope effects alone has revealed that in the transition state the C—D bond is largely broken, the central carbon bears substantial negative charge and is probably well on its way to a trigonal configuration, and that the reaction proceeds largely with retention of configuration. These results represent a unique example of the power of deuterium isotope effects as a tool in theoretical chemistry.

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* I am indebted to D. P. Stevenson of the Shell Development Company, Emeryville, Calif., for these analyses.

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SECONDARY DEUTERIUM ISOTOPE EFFECTS IN CHEMICAL AND BIOCHEMICAL REACTIONS*

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Introduction

The treatments of Bigeleisen[1] and Melander[2] both show that the effect of isotopic substitution on reaction rate can be analyzed in terms of differential changes of molecular vibrational frequencies associated with the process of conversion of starting molecules into the transition state configuration. The largest effects, particularly with the isotopes of hydrogen, can be associated with changes in those frequencies considered to be almost exclusively vibrations of the isotopic atom itself. In general, since frequencies of the starting molecules are subject to independent experimental observation while those of the transition state are not, the former are usually used in connection with studies of the isotope rate effects in reaction mechanism to provide evidence of transition-state vibration frequencies and therefore of transition-state structure.

The largest changes in isotopic atom vibration frequencies during reaction and therefore the largest isotope rate effects are observed in those reactions that involve the transfer of the bonding of the isotopic atom from one atom in the initial state to another in the intermediate or final state. For the deuterium-hydrogen case such primary isotope rate effects frequently involve rate ratios for the isotopic molecules of as much as seven to ten.

Experimental observations of recent years have made convenient the definition of a secondary deuterium isotope rate effect as an effect on the rate of a reaction caused by substitution of deuterium for a hydrogen that is not being transferred in the rate controlling step.

Although certainly a part of most (if not all) isotope effects due to a change of solvent from water to deuterium oxide would be classified as "secondary" by this definition, in this paper we restrict ourselves to a consideration of those rate effects, in mechanistically fairly well-defined reactions, which are due to isotopic substitution at a limited number of specific sites in the reacting compounds.

In principle, isotope effects due to those changes in frequencies of complex vibrations that cannot be accurately regarded as simple vibrations of the isotopic atom are possible but these in general would be expected to be small. Most of the important secondary isotope rate effects that have been observed in organic chemistry are large enough to represent changes in the vibration frequencies of the isotopically substituted atom itself during the activation process and therefore indicate some intramolecular interaction between the reaction

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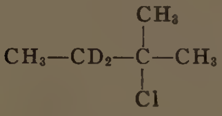
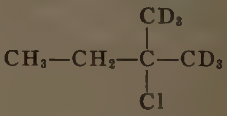
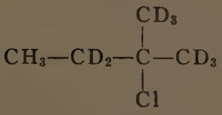
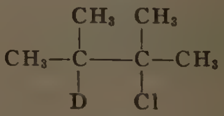
† Alfred P. Sloan Research Fellow.

‡ National Science Foundation Predoctoral Cooperative Fellow.

site and the site of isotopic substitution to account for the frequency changes at the latter.

Thus the aim of most reaction mechanism studies using secondary isotope effects has been to elucidate the structure of the transition state and the nature of any intramolecular interaction between the reaction center and the site of isotopic substitution. In this paper we shall first review, as an example, the information concerning the most carefully studied kind of secondary isotope rate effect: that caused by deuterium substitution on the carbon atom next to the reaction center in carbonium ion type reactions. We shall then summarize some other examples of secondary deuterium isotope effects in organic chemistry and finally present some new results on isotope effects in some enzyme-catalyzed hydrogen-transfer reactions that we hope will help provide a bridge between the chemical and biological parts of this interdisciplinary monograph.

TABLE 1
TERTIARY ALKYL CHLORIDE SOLVOLYSES
80 Per cent Aqueous Alcohol, 25° C.

 $CH_3-CD_2-\underset{Cl}{\overset{CH_3}{C}}-CH_3$	 $CH_3-CH_2-\underset{Cl}{\overset{CD_3}{C}}-CD_3$
k_H/k_D 1.40*	1.77*
 $CH_3-CD_2-\underset{Cl}{\overset{CD_3}{C}}-CD_3$	 $CH_3-\underset{D}{\overset{CH_3}{C}}-\underset{Cl}{\overset{CH_3}{C}}-CH_3$
k_H/k_D 2.35*	1.28†

* From Shiner.[4]
† From Shiner.[5]

β-Deuterium Isotope Effects in Carbonium Ion Reactions

These effects were first observed several years ago by Lewis and Boozer[3] and by Shiner.[4] TABLE 1 presents some isotope effects in the solvolysis of β-deuterated *t*-alkyl chlorides.[4]

The theory discussed above suggests that these effects must be due to a loss of vibrational frequency on activation of the isotopically substituted hydrogen atoms. Since the effect is cumulative as β-deuterium substitution increases, this frequency loss apparently is characteristic of all hydrogen atoms attached to the β-carbon. The mechanism of these solvolyses has been extensively studied[6] and it is believed that the rate-determining step of the reaction involves separation of the chlorine-carbon bond into the ionic fragments, chloride ion, and carbonium ion. In this process the β-H atoms are not being transferred and therefore this is classified as a secondary isotope rate effect.‡

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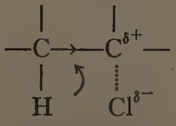
‡ Since these reactions lead eventually in the second step to production of some olefin as well as the substitution products (tertiary alcohol and ether) the question arises as to whether the generally accepted mechanism for these processes is wrong and the isotope effect is ac-

Making certain reasonable approximations and assuming that the hydrogen vibrations concerned are harmonic, so that the vibration frequency of hydrogen in any bond is about 1.36 times that for deuterium in the same bond, the net frequency loss per C—H bond in the transition state needed to account for these isotope effects is about 100–300 cm.$^{-1}$. A saturated aliphatic C—H bond generally has a stretching frequency near 2900 cm.$^{-1}$, and two bending frequencies near 1500 cm.$^{-1}$. Thus, although the proportional change in frequency is small it is nevertheless larger than the variations normally encountered in comparing C—H stretching frequencies in normal aliphatic molecules and apparently indicates some special interactions in the carbonium ion-forming transition state.[7]

Transition-State Model for Solvolysis

One is therefore presented with the problem as to what sort of interaction takes place between the reaction center and the β-hydrogens to cause the latter to change vibration frequency on activation. Physical-organic chemists recognize several types of intramolecular interactions that might be operative in this substitution:



(1)

Referring to the figure representing the transition-state model for solvolysis (1), these can be enumerated as follows:

(1) The developing positive charge on the carbon atom (δ^+) can exert an electron-attracting inductive effect (→), through its bond to the β-carbon, on the β-C—H bond.

(2) The developing vacant orbital on the α-carbon might overlap directly with the β-CH bond, giving a hyperconjugative transfer (↷) of electron density. In the extreme this could become a form of neighboring group participation.

(3) The departing chloride ion and the accompanying spatial rearrangement around the α-carbon might release some nonbonded repulsions between these atoms and the β-H atom.

(4) There may be specific interactions between the β-situated hydrogen atoms and adjacent solvent molecules enhanced by the polar character of the transition state.

(5) The strong carbon-chlorine dipole created in the transition state may, by a direct electrostatic field effect, influence the vibration frequency of the adjacent hydrogen atoms.

It is obviously not an easy matter even to decide which of the above effects predominates, much less to determine quantitatively the degree of contribution

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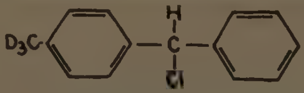
tually a primary effect on a direct elimination reaction. This hypothesis does not bear up under detailed examination. The difficulty with it is most clearly shown in the hexa-deutero *t*-amyl chloride example. Since most of the elimination in the solvolysis of *t*-amyl chloride takes place toward the methylene carbon to give trimethylethylene this compound yields very nearly the same proportion of olefin as *t*-amyl chloride itself. Yet it shows the characteristic β-deuterium isotope effect.

of each. However, by using the classic organic chemical approach of comparing rate effects and other properties among a large number of compounds with specific known structural relationships considerable restrictions have been placed upon the nature of any explanation of these phenomena. On the basis of the rather limited evidence then available it was tentatively suggested early in the investigation of these isotope effects that they could be correlated best with the suggestion (No. 2, above) that hyperconjugation interactions were the dominating influence.[3,4] Subsequent experimental information has seemed to confirm this original suggestion.

It was reasoned that an inductive effect in this reaction would lead essentially to increased electronegativity of the β-C atom, increased C—H bond strength, and to an increase in the CH frequency rather than to the decrease required to account for the experimental results. This rather crudely reasoned explanation of the direction of inductive effects on deuterium isotope effects seems to be confirmed (see below) in experiments where hyperconjugation interactions are structurally prohibited. The nonbonded-repulsion, or release-of-strain, argument was discounted because aliphatic C—H stretching and bending frequencies seem to be remarkably independent of the steric nature of groups attached to adjacent carbon atoms. "The symmetrical deformation mode of the hydrogen atoms of a methyl group results in an absorption band in the range 1385–1370 cm.$^{-1}$ which is extremely stable in position provided that the methyl group is attached to another carbon atom."[8] For aliphatic hydrocarbons the range is that given above while in methyl acetylene this frequency is 1379 cm.$^{-1}$, still within the same narrow range. In order to test further the importance of direct steric interactions in these effects Shiner and Verbanic[9] resorted to the classical organic method of distinguishing electronic from steric effects. The former are considered to be transmitted in diminished intensity through intervening unsaturated linkages while the latter are eliminated since the group to be investigated is now removed from the direct vicinity of the reaction center. The observation that *p*-trideuteromethyl benzhydryl chloride still solvolyzed more slowly than its protium analog by a factor of 1.06 indicates that direct steric interactions do not alone suffice to explain the results.



p-trideuteromethylbenzhydryl chloride

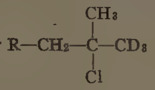
Streitwieser and his co-workers[10] showed that the solvolysis of cyclopentyl toluenesulfonate was slowed almost as much by *trans* (k_H/k_D = 1.16) as by a *cis* (k_H/k_D = 1.22) β-deuterium atom. If the direct field effect mentioned above were the most important factor it certainly would exhibit a markedly larger influence on the frequency of the much closer *cis* hydrogen.

In another series of experiments (V. J. Shiner, unpublished results)[11] the effect of various β-alkyl substituents on the β-deuterium secondary isotope effects on tertiary alkyl halide solvolyses were investigated giving the results summarized in TABLE 2.

TABLE 2

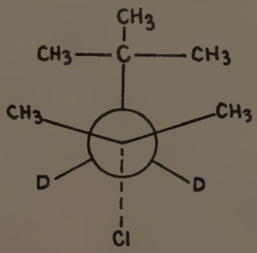
TERTIARY ALKYL CHLORIDE SOLVOLYSES

80 Per cent Aqueous Alcohol at 25° C.*

R	k_H/k_D for  $R\text{—}CH_2\text{—}\overset{CH_3}{\underset{Cl}{C}}\text{—}CD_3$	k_H/k_D for $R\text{—}CD_2\text{—}CCl(CH_3)_2$
Me	1.34	1.40
Et	—	1.37
i-Pr	1.34	1.44
t-Bu	1.40	1.08

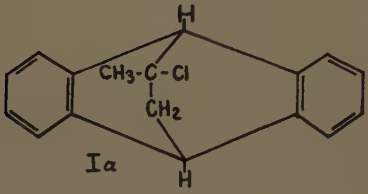
* V. J. Shiner[11] and unpublished results.

The important thing to note from this series of results is that all but one of these compounds shows the expected normal β-deuterium isotope effect. This particular example that has one β-carbon atom bearing a *t*-butyl group and two deuterium atoms shows an isotope effect about five times smaller than normal. This unusual result can be accommodated by, and provides support for, the hyperconjugation hypothesis if it is assumed that the transition state has a conformation shown in a Newman projection formula as follows:

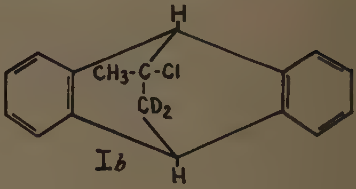


In this formula the central dots represent the α-carbon and the circle the β-carbon atom, with the attached groups oriented as shown. This conformation would be expected to be favored because the bulky β-*t*-butyl group would prefer the upper position that has least steric hindrance. This would force the β-C—D bonds into the positions shown where they are nearly perpendicular to the developing vacant orbital on the α-carbon atom. In these positions they are not favorably oriented for maximum hyperconjugative overlap with the developing vacant orbital; the requirement for maximum overlap is that the overlapping orbitals be parallel. Thus with diminished hyperconjugation there is diminished interaction between isotopic and reaction centers and a smaller isotope effect.

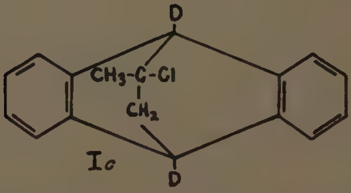
The one unsatisfactory aspect of this explanation is that there is no independent way to establish the conformation of the transition state. Therefore a more unambiguous way of determining this orientational influence on the isotope effect was sought. Accordingly the bicyclic compounds, *Ia*, *b*, *c* were synthesized and their solvolysis rates determined.[12]



Ia



Ib



Ic

The cage nature of the bicyclic ring system ensures that the so-called bridgehead C—H bond at the top of the formula is directed in space perpendicular to those orbitals of the adjacent carbon atom that are not involved in the formation of the ring. Thus the hyperconjugation explanation of the β-deuterium isotope effect on solvolysis rates requires that compound I*c* solvolyze no slower than I*a*. The observed k_H/k_D ratio was 0.985. No such spatial restrictions apply to compound I*b* that as expected showed a more nearly normal k_H/k_D ratio of 1.15. It is believed that these results provide a quantitative comparison of the relative magnitude and direction of inductive and hyperconjugative influences on these β-deuterium secondary isotope effects in carbonium ion reactions. Thus electron attraction from a CH bond by the inductive effect seems to cause a very small isotope effect making the deuterium compound react slightly faster. Hyperconjugative withdrawal causes a much larger effect in the opposite direction.

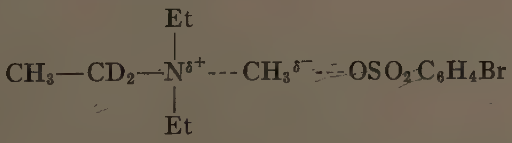
Other Secondary Isotope Effects

Streitwieser and his co-workers[10] as well as Johnson and Lewis[13] and Saunders and his co-workers[14] have observed that α-deuterium substitution also causes a slowing of a carbonium ion solvolysis reaction generally by about 15 per cent. Stewart *et al.*[15] observed similar effects in carbonium ion formation equilibrium constants. This is not unexpected because the isotopic atom, although it is not being transferred in the rate-determining step, is nevertheless attached directly to a carbon atom that is undergoing reaction. The bond to this hydrogen is changing hybridization during the course of the reaction and will certainly be expected to show alterations in its vibration frequencies. Streitwieser[10] has suggested that it is the initial state bending frequency that becomes the out-of-plane bending frequency in the transition state that is most drastically lowered.

Lewis and Johnson[13] and also Lewellyn *et al.*[16] have observed that trideuteromethyl derivatives solvolyze (apparently by a direct displacement reaction rather than via a carbonium ion) more rapidly than their protium analogs. Lewis and Johnson have made the very interesting suggestion that this indicates that the α-deuterium isotope effect may be a measure of the degree of nucleophilic participation in a displacement reaction, showing k_H/k_D values greater than one in carbonium ion reactions and less than one in direct displacements. It is perhaps still too soon to assess the generality and usefulness of this as a criterion of mechanism because the only k_H/k_D values less than one that have been observed in this series pertain to reactions of the trideuteromethyl compounds, all of which show k_H/k_D values from 0.87 to 1.00. The suggested explanation for the α-deuterium isotope effect being greater than one in a direct displacement reaction is that steric crowding of the α-hydrogens in the transition state between the incoming and leaving groups increases their bending vibrational frequencies. In the carbonium ion transition state the absence of crowding by the incoming group allows a lower bending frequency.

Halevi[17] has observed that α-deuteration of phenylactic acid and of the benzyl ammonium ion leads to increased acidities in both compounds. He attributes these results to differences in ground state electron distributions dependent on anharmonicity influences. Although anharmonicity effects may make the estimation of frequencies difficult there are apparently no large anharmonicity influences on isotope rate or equilibrium effects outside of those exerted through the molecular vibrational frequencies. Where the vibrational frequencies are accurately known in both reaction states no extra effects of anharmonicity need apparently be included to quantitatively account for the observed isotope effects.[18] Halevi's results correlate with the suggestion made above that increased electron attraction by the inductive effect increases the vibrational frequencies of the hydrogen compound more than those of the deuterium compound. This causes the deuterium compound to show larger rate or equilibrium constants for the transition to the more electron-demanding situation.

An apparently related effect has been observed by Lewis[19] in the reaction of α-dideuterotriethyl amine with methyl bromobenzene sulfonate. In the transition state for this displacement reaction, the inductive withdrawal



$$CH_3{-}CD_2{-}\overset{\displaystyle Et}{\underset{\displaystyle Et}{N}}{}^{\delta+}\text{- - -}CH_3{}^{\delta-}\text{- - -}OSO_2C_6H_4Br$$

of electrons from the β-CD bonds is increased due to the developing positive charge on the nitrogen atom. The resulting k_H/k_D ratio of 0.93 finds a satisfactory explanation by the general hypothesis regarding inductive effects outlined above.

Denny and Tunkel[20] have observed isotope effects in a number of addition reactions to *trans*-1,2-diphenylethylene-1,2-d$_2$ and found the k_H/k_D ratio to be near 0.90 in all cases. The explanation of these results is apparently the reverse of that supplied by Streitwieser for the α-effect on solvolysis reactions. The results of Cross and Shiner[21] on the effect of α-deuteration on the hydrolysis rates of ketals as well as those obtained by Bender *et al.*[22] on the addition reactions of α-deuterated carbonyl compounds are in accord with the hyperconjugation explanation used above. The results of Hodnett *et al.*[23] on the hydrolysis of methyl *p*-methyl-*t*-benzoate require, in a way consistent with the ideas outlined above, both inductive and hyperconjugative contributions to the isotope effect. Winstein and Takahashi[24] have shown that in a solvolysis reaction believed to proceed by neighboring hydrogen participation, replacement of that neighboring hydrogen slows the solvolysis rate considerably more than in the cases cited above where participation is not important. Thus 3-methyl-2-butyl-3-*d* *p*-toluenesulfonate, or $(CH_3)_2CD—CH(CH_3)OTS$, solvolyzes slower than its protium analog by a factor of about two. This participation can be viewed as an extreme form of hyperconjugation.

The isotope effect observed on carbonium ion reactions by deuteration of alkyl groups attached to benzene rings are solvent sensitive and apparently are not reliable simple guides to relative degrees of hyperconjugation.[9,25,26]

Isotope Effects in Enzyme-Catalyzed Hydrogen Transfer Reactions

In this study we have examined the reaction between diphosphopyridine-nucleotide (DPN) and ethanol to give the reduced nucleotide (DPNH) and acetaldehyde, and also the reverse process. We have made use of two different enzymes that catalyze this process: yeast alcohol dehydrogenase and liver alcohol dehydrogenase. By examining the rate effect of deuterium substitution at the site of attachment of the transferrable hydrogen we have endeavored to obtain information regarding the nature of the binding of these hydrogens in the intermediate and transition states in these reactions and thus to elucidate the intimate details of the mechanism.

Kinetic studies on these systems have previously been reported by Theorell and Chance,[27] Hayes and Velick,[28] Nygaard and Theorell,[29] Theorell *et al.*,[30] Dalziel,[31] and Mahler and Douglas.[32]

These reactions apparently involve coordination of the coenzyme and/or substrate to the enzyme followed by hydrogen transfer and subsequent dissociation of the products.

If the reactions for complexing and dissociation of both forms of the substrate and coenzyme to the enzyme are rapid compared to the hydrogen transfer, an equilibrium mechanism applies as follows:

$$E + Co\mathrm{H} \rightleftharpoons E\cdot Co\mathrm{H} \qquad (K_1)$$

$$E + S \rightleftharpoons E\cdot S \qquad (K_2)$$

$$E\cdot Co\mathrm{H} + S \rightleftharpoons E\cdot Co\mathrm{H}\cdot S \qquad (K_3)$$

$$E\cdot S + Co\mathrm{H} \rightleftharpoons E\cdot Co\mathrm{H}\cdot S \qquad (K_4)$$

$$E\cdot Co\mathrm{H}\cdot S \xrightarrow{k} E\cdot Co\cdot S\mathrm{H} \qquad k$$

The transfer step is then followed by a further symmetrical set of equilibrium reactions for dissociation of the oxidized coenzyme and reduced substrate from the enzyme. In these equations E represents enzyme, CoH reduced coenzyme, S oxidized substrate, Co oxidized coenzyme, SH reduced substrate, and $E\cdot Co$H an enzyme-coenzyme complex. Alberty[33] and Dalziel[31] have shown that if all steps except the actual hydrogen transfer are equilibria then the following general initial rate equation[*] applies:

$$\frac{(E_0)}{v_i} = \frac{1}{k} + \frac{K_4}{k\,(\mathit{Co}\mathrm{H})} + \frac{K_3}{k(S)} + \frac{K_1K_3}{k\,(\mathit{Co}\mathrm{H})\cdot(S)}$$

where v_i is the initial rate and the brackets represent molar concentrations. If the binding steps are not rapid enough to achieve equilibrium the mechanism can be represented in a general way as follows:

$$E + \mathit{Co}\mathrm{H} \xrightleftharpoons[k_2]{k_1} E\cdot\mathit{Co}\mathrm{H}$$

$$E\cdot\mathit{Co}\mathrm{H} + S \xrightleftharpoons[k_4]{k_3} E\cdot\mathit{Co}\mathrm{H}\cdot S \xrightleftharpoons[k_6]{k_5} E\cdot\mathit{Co}\cdot S\mathrm{H} \xrightleftharpoons[k_8]{k_7} E\cdot\mathit{Co} + S\mathrm{H}$$

$$E\cdot\mathit{Co} \xrightleftharpoons[k_{10}]{k_9} E + \mathit{Co}$$

Dalziel[31] has shown that for this situation the steady state assumption leads to the following initial rate laws.
Forward direction:

$$(E_0)/v_i = \phi_0 + \frac{\phi_1}{(\mathit{Co}\mathrm{H})} + \frac{\phi_2}{(S)} + \frac{\phi_{12}}{(\mathit{Co}\mathrm{H})\cdot(S)}$$

Reverse direction:

$$(E_0)/v_i = \phi_0' + \frac{\phi_1'}{(\mathit{Co})} + \frac{\phi_2'}{(S\mathrm{H})} + \frac{\phi_{12}'}{(\mathit{Co})\cdot(S\mathrm{H})}$$

where the "ϕ" constants represent complex combinations of the rate constants as follows:

$$\phi_0 = \frac{1}{k_9} + \frac{1}{k_7} + \frac{1}{k_5} + \frac{k_6}{k_5k_7}$$

$$\phi_1 = 1/k_1$$

$$\phi_2 = \frac{k_4k_7 + k_4k_6 + k_5k_7}{k_3k_5k_7}$$

$$\phi_{12} = k_2/k_1\cdot\phi_2 \doteq K_1\phi_2$$

Similar expressions apply for the "ϕ'" constants for the reverse reaction. It is important to note that the general form of the initial rate law is the same whether the equilibrium or steady state situation[†] applies. It is apparent

[*] It is apparent that this equation is of the general form[31]

$$\frac{(E_0)}{v_i} = \phi_0 + \frac{\phi_1}{(\mathit{Co}\mathrm{H})} + \frac{\phi_2}{(S)} + \frac{\phi_{12}}{(\mathit{Co}\mathrm{H})(S)}$$

[†] Certain simpler mechanisms, that is, ones postulating only 3, 2, or 1 enzyme-containing complexes instead of the 4 shown above also lead to the same rate law. This is accomplished by setting the appropriate *k*s equal to infinity.

therefore that a variety of different mechanisms can account in a satisfactory manner for the empirically observed linear dependence of reciprocal rates on the reciprocal of the concentrations of substrate and coenzyme added. In order to make any progress in an understanding of the function of the enzyme, the nature of the interaction of the enzyme with coenzyme and/or substrate and of the transition state in the hydrogen transfer step proper, it is clearly necessary to have available as a first step methods for distinguishing at least different classes of mechanisms (that is, whether equilibrium or steady state). Some kinetic criteria for this purpose have been suggested by Dalziel,[31] while Alberty has proposed the use of relationships between kinetic parameters and the equilibrium constant for the over-all reaction on one hand[33] and a study of the effect of adding the reaction products on the kinetic parameters on the other.[34] We are applying these tests to the reactions under study, but their routine use requires measurements on the reaction in both directions and proves cumbersome and somewhat lacking in precision. Considerable utility attaches therefore to the development of additional, simple, and easily applied means to the same end. We believe that studies on isotope rate effects may provide this means. By making certain reasonable assumptions, that is, that there be isotope effects of different magnitude on reactions involving the rate of dissociation of an isotopically substituted substrate or coenzyme to the enzyme and on the rate of hydrogen transfer:* it is easy to show that for the equilibrium mechanism,

$$\bar{\phi}_0 \equiv \bar{\phi}_2 ;\quad \bar{\phi}_1 \equiv \bar{\phi}_{12} \qquad \text{and} \qquad \bar{\phi}_0{}' \equiv \bar{\phi}_1{}' ;\quad \bar{\phi}_2{}' \equiv \bar{\phi}_{12}'$$

while for the steady state mechanism only the following relationships obtain:

$$\phi_1 = 1; \qquad \bar{\phi}_2{}' \equiv \bar{\phi}_{12}'$$

Here the various $\bar{\phi}$ represent the isotope effects on the respective ϕ, that is, ϕ^D/ϕ^H.

Following the method of Theorell and Chance[27] the initial rates were determined with a recording ultraviolet spectrophotometer taking advantage of the difference in optical density between DPN and DPNH at 340 mμ. The coefficients of the rate equation (that is, the ϕ's) given above can be determined by the graphical method of Dalziel[31] in which for a given enzyme concentration the reciprocals of the initial velocities are plotted against the reciprocals of the substrate concentration at various coenzyme concentrations. This gives a straight line for each coenzyme concentration that has for the forward reaction a slope $\phi_1 + [\phi_{12}/(S)]$ and an intercept of $\phi_0 + [\phi_2/(S)]$. If these slopes for different substrate concentrations are plotted against the reciprocals of the substrate concentration, a straight line of slope ϕ_{12} and intercept ϕ_1 is obtained. If the intercepts of the original plot are plotted against reciprocals of the substrate concentrations then a straight line of slope ϕ_2 and intercept ϕ_0 is obtained. Thus from the double reciprocal plots ϕ_0, ϕ_1, ϕ_2, and ϕ_{12} can all be obtained.

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* That is, an isotope effect of magnitude α for the former and of magnitude β for the latter; the same results, however, are obtained in the more general case of an isotope effect α for a rate of association, one of magnitude β for a rate of dissociation, one of magnitude γ for the hydrogen-transfer step, all in the forward direction: and of magnitude α', β', and γ' in the reverse directions, even if $\alpha \neq \alpha'$.

These can also be obtained in an equivalent way as a check on the method by first plotting the same initial rate reciprocals against the reciprocals of the substrate concentration at constant coenzyme concentration. Similar treatment of initial rate data for the reverse reaction yields the ϕ' constants.

For the calculation of isotope effects this graphical method of determining the rate constants is not particularly satisfactory because it is of limited accuracy and gives no error estimate. Therefore we have developed an IBM 650 electronic computer program for determining the slopes and intercepts of the lines using a Gaussian least squares method. Thus no subjective error is involved in the data treatment and an error estimate is provided with the results. The program can accommodate a matrix of 10 substrate by 10 coenzyme concentrations (that is, a total of 100 rate runs) and the time per entry is 2 sec. (that is, 200 sec. for the complete matrix).

The yeast alcohol dehydrogenase reaction has been studied in this work only in the direction involving oxidation of the coenzyme and reduction of acetaldehyde. The reduced coenzyme has two hydrogens attached to the C_4 position of the nicotinamide ring. These two hydrogens are not configurationally equivalent and only one, called "α," is transferred in this highly stereospecific reaction.[35,36] We have stereospecifically synthesized the "α"-deuterium compound[35] and determined the effect of this isotopic substitution for the transferred hydrogen on the kinetic parameters of the reaction. It was easy to observe that the over-all reaction rate with the deuterium compound was slowed down by a factor of about two. However, considerable errors are involved in the reduction of this observed effect into effects on the individual reaction rate constants.

Application of the isotopic rate criteria just described for mechanism selection suggests that under the particular conditions studied (0.01 *M* phosphate, *p*H 7.45, $t = 24°$), the yeast alcohol dehydrogenase reaction obeys the equilibrium mechanism: $\bar{\phi}_0 = 1.75 \pm .07$; $\bar{\phi}_1 = 1.31 \pm .17$; $\bar{\phi}_2 = 2.00 \pm .36$; $\bar{\phi}_{12} = 1.40 \pm .14$. Thus $\phi_0 \equiv \phi_2$; $\bar{\phi}_1 \equiv \bar{\phi}_{12}$.

It is of interest to note that Dalziel[32] has reached essentially the same conclusions from an application of his criteria to the data of Nygaard and Theorell.[30]

This then permits us to calculate the various equilibrium constants involved and to establish the nature and magnitude of the isotope effect on these as well as on the rate constant for the hydrogen transfer step. These data are summarized in TABLE 3.

It should be noted first that the primary isotope effect for the transfer step, 1.75, is relatively small, indicating a high degree of conservation of zero point energy in the transition state for this reaction. Second, the binding-dissociation constants for acetaldehyde are well within the estimated limits of error, the same, whether the reaction is with a hydrogen or deuterium carrying coenzyme. Third, and most surprising, there is a difference in the binding-dissociation constants for the deuterated and nondeuterated coenzymes. These numbers are difficult to determine accurately, and the error limits are rather large, but all comparisons and tests that we have been able to make indicate that these isotope effects on the binding-dissociation constants are real. For instance, essentially the same effect is obtained on both K_1 and K_4; that is, the isotope effect on the coenzyme binding is independent of the substrate binding. It would seem that these are much too large to be simply secondary effects caused by electronic interaction between the site of isotopic substitution and some enzyme binding site at another part of the coenzyme molecule. It would therefore appear that the hydrogen that is to be transferred undergoes some kind of direct interaction with the enzyme in the binding step raising its vibration frequencies about 300 to 400 cm.$^{-1}$ This is obviously a point of considerable mechanistic significance.

In the reaction catalyzed by liver alcohol dehydrogenase we have examined the isotope effect on the kinetic constants of both α- and β-deuterium substitution in the reduced coenzyme. This reaction appears to follow the steady state mechanism and the binding dissociation equilibrium constants can be obtained from the ratio ϕ_{12}/ϕ_2. For replacement of the α-hydrogen by deuterium the over-all rate is slowed down by a factor of about 2.0 and the ratio for the binding dissociation constants for the coenzyme and α-deuterated coenzyme, that is, K_H/K_D is 1.22 ± 0.12, very near to that obtained in the yeast alcohol dehydrogenase example. This then indicates again a direct interaction between the enzyme and the hydrogen of the coenzyme that is to be transferred, which increases the frequency of this hydrogen in the bound state by about 300 cm.$^{-1}$. In addition ϕ_1 gives the binding rate constant k_1 and the isotope effect on the binding rate, k_{1H}/k_{1D}, can be calculated. This was found to be about 1.6 ± 0.2 indicating that although the hydrogen compound is bound less tightly than the deuterated analog at equilibrium, it is bound more rapidly. In terms of total vibration frequency changes, these hydrogens must lose about 800 to 900 cm.$^{-1}$ more in going to the bound state. The story for the β-deuterium isotope effect is different and very interestingly so. The β-deuterated coenzyme is bound less tightly than the hydrogen analog, showing a dissociation constant ratio, k_H/k_D, of about 0.56. The rate of binding is about the same for the β-D compound as for the undeuterated one. Thus the β-H apparently suffers no net change in frequency in passing from the unbound state to the transition state, but then loses about 900 cm.$^{-1}$ in going to the bound state. Unfortunately the kinetic law does not allow us to calculate the transfer constant k_6 directly so that we cannot estimate the primary isotope affect in this reaction, or the β-deuterium secondary effect in the α-hydrogen transfer steps.

TABLE 3

CONSTANTS FOR REACTIONS OF DPNH AND α-DPND WITH ACETALDEHYDE CATALYZED BY YEAST ALCOHOL DEHYDROGENASE

All in Moles/l.

Constant	Binding of	DPNH	α-DPND	K_H/K_D
K_1	coenzyme	$8.3 \pm 1.0 \times 10^{-6}$	$5.8 \pm 1.0 \times 10^{-6}$	1.43 ± 0.34
K_4	coenzyme	$8.43 \pm 0.43 \times 10^{-5}$	$6.29 \pm 0.68 \times 10^{-5}$	1.34 ± 0.21
K_2	acetaldehyde	$5.44 \pm 0.32 \times 10^{-5}$	$5.81 \pm 0.91 \times 10^{-5}$	0.93 ± 0.20
K_3	acetaldehyde	$5.55 \pm 0.67 \times 10^{-4}$	$6.35 \pm 0.63 \times 10^{-4}$	0.88 ± 0.19
k	(*transfer step*)	2.05×10^4 sec.$^{-1}$	1.17×10^4 sec.$^{-1}$	$k_H/k_D = 1.75$

It seems again, however, that the primary effect is in the normal direction and somewhat small, being near to 1.5 or 2.0.

These results are sufficiently novel and the errors in the original experimental measurements are so magnified in their reduction to the individual reaction rate or equilibrium constant that our conclusions at this time must be regarded as tentative. However, great care has been taken to eliminate all sources of error and the isotope effects that we have reported seem to be real. It further appears that they are large enough so that some of them can be checked by completely independent nonkinetic experiments. Such further work is now in progress in our laboratories.

Our tentative conclusions are, then, that in the binding steps of the reduced coenzyme to the enzyme there is direct interaction between the enzyme and the hydrogen to be transferred. This process involves a smaller and different kind of effect on the other hydrogen that is attached to the same site, but is not transferred.

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ACID-BASE STUDIES WITH DEUTERIUM OXIDE*

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Very soon after the preparation of nearly pure deuterium oxide, reports began to appear on studies of rates and equilibria in this new solvent. These early studies showed that acid strengths were commonly less in D_2O than in H_2O by factors of from 2 to 5 and, consistently, that rates of acid- and base-catalyzed reactions were frequently greater in the solvent D_2O. The fact that there were some sizeable differences in the observed rate ratios between the solvents D_2O and H_2O, and that these differences correlated with changes in reaction type, made these results of considerable interest to investigators of kinetic mechanisms. This interest has continued to the present. From a more general standpoint, however, one of the considerable virtues of deuterium oxide as a solvent is that it is almost, but not quite, like water. Hence by making relative studies in D_2O one can often throw light on aspects of the more important solvent, water. This is true of acid-base reactions, along with many others.

The fields of acid-base equilibria and acid-base catalysis are immense ones and both have had extensive discussion in reviews and monographs, a few examples being the books of Bell,[1,2] Gurney,[3] and Frost and Pearson.[4] These treatments give ample coverage to the earlier literature. Hence it seems appropriate to restrict the present discussion to recent developments, with emphasis on the particular contributions of studies in D_2O to problems of acid-base catalysis.†

One question of interest is the extent to which relative studies in D_2O can give information on mechanism. For either acid or base catalysis a variety of mechanisms is possible, so that it is necessary to consider the results for each of them to see if the differences are significant. The following is a list of various mechanisms for the particular case of acid catalysis:

(1) Specific catalysis by solvated proton
(2) General acid catalysis
    (*a*) Pre-equilibrium formation of conjugate acid (slow step is proton removal by base)
        (*1*) Slow exchange of proton removed
        (*2*) Fast exchange of proton removed
    (*b*) Single-step proton transfer
    (*c*) Formation of H-bonded complex

A very similar list can be written down for base catalysis, and for each class it is possible to formulate some of the reactions either by using termolecular formulations or successive bimolecular steps. Detailed discussion will be restricted to acid catalysis and will be given in terms of the more familiar bi-

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† Much of the discussion of this section is based on a more detailed treatment given recently.[5]

molecular mechanisms. For brevity, the rate laws will be given in concentration units and, where pre-equilibria enter, it will be assumed that only small amounts of conjugate acid have formed.

Specific hydrogen ion catalysis. True specific hydrogen ion catalysis occurs only if there is a pre-equilibrium proton transfer with a rate-determining step that does not involve another proton transfer. Using S for substrate we have

$$S + \mathrm{H}X = S\mathrm{H}^+ + X^- \qquad \text{(equilibrium)}$$

$$S\mathrm{H}^+(+M) \xrightarrow{\mathrm{k}_s} \text{Products} + \mathrm{H}^+ \qquad \text{(slow)}$$

where HX may be either a strong acid, that is, H_3O^+, or a weak acid.

If we choose a unimolecular slow step and consider first the case of a strong acid catalyst, we can write the rate v as

$$v = \mathrm{k}_s C_{S\mathrm{H}^+} = \frac{\mathrm{k}_s}{\mathrm{K}_{S\mathrm{H}^+}} C_S C_{\mathrm{H_3O}^+} \tag{1}$$

where the symbol C_S refers to concentration of substrate and $C_{\mathrm{H_3O}^+}$ to total hydrogen ion concentration, that is, total concentration of strong acid.

Equation 1 also holds if the pre-equilibrium is established with a buffer mixture. In this case

$$v = \frac{\mathrm{k}_s \mathrm{K}_{\mathrm{H}A}}{\mathrm{K}_{S\mathrm{H}^+}} \frac{C_{\mathrm{H}A} C_S}{C_{A^-}} = \frac{\mathrm{k}_s}{\mathrm{K}_{S\mathrm{H}^+}} C_S C_{\mathrm{H_3O}^+} \tag{2}$$

However, in considering relative rates in H_2O and D_2O for the buffered case, one must be careful to distinguish between two possible experimental situations, the one in which hydrogen ion concentration is maintained constant for the two solvents and the one in which the buffer ratio is maintained constant. For the strong acid case the first of these is obviously the normal situation.

Equation 1 shows that the second-order rate constant k^{H} for reaction of substrate and hydrogen ion is really composite and is given by $\mathrm{k}_s^{\mathrm{H}}/\mathrm{K}_{S\mathrm{H}^+}$, where the superscript H specifically denotes reaction in the solvent water. A similar equation will hold for reaction in solvent deuterium oxide. Hence the ratio of the second-order rate coefficients for the two solvents will be

$$\frac{\mathrm{k}^{\mathrm{D}}}{\mathrm{k}^{\mathrm{H}}} = \frac{\mathrm{k}_s^{\mathrm{D}}}{\mathrm{k}_s^{\mathrm{H}}} \frac{\mathrm{K}_{S\mathrm{H}^+}}{\mathrm{K}_{S\mathrm{D}^+}} \tag{3}$$

To predict the variation in relative rate with change in solvent from H_2O and D_2O one must thus consider the solvent effect on two ratios, $\mathrm{k}_s^{\mathrm{H}}/\mathrm{k}_{\mathrm{D}}^{\mathrm{H}}$ and $\mathrm{K}_{S\mathrm{H}^+}/\mathrm{K}_{S\mathrm{D}^+}$. The former of these will be normally close to unity, varying perhaps from 0.7 to 1.1. The reasons are that there will normally not be any *kinetic* isotope effect for the slow step and, since this reaction cannot be a proton transfer, the only variation in rate will result from general medium effects that are expected to be small. In contrast, the ratio of ionization constants $\mathrm{K}_{S\mathrm{H}^+}/\mathrm{K}_{S\mathrm{D}^+}$, for the conjugate acids that are formed in the first step, will be normally rather large, varying from perhaps 2 to 4. This is simply a reflection of the usual relative weakness of acids in D_2O, a fact that has been known for some time for weak acids[6] and that has recently been shown also to be true for quite strong acids.[7] The over-all expectation is, then, that for this mechanism the observed ratio k^D/k^H will be greater than unity, varying from perhaps 1.4 to 3. Typical data for reactions that follow this mechanism are shown in TABLE 1, which illustrates that the data agree with expectation.

If an acid-catalyzed reaction of this type is studied in the solvents H_2O and D_2O under the condition of constant buffer ratio, rather than that of constant concentration of hydrogen ion, the predicted ratios of rate coefficients are somewhat different. From Equation **2** we can write

TABLE 1

EXPERIMENTAL RESULTS FOR REACTIONS WITH PRE-EQUILIBRIUM PROTON TRANSFER[8-14]

Reaction	Spec.	Gen.	k^D/k^H	Ref.
Methyl acetate hydrolysis	H^+		1.6	8
Acetamide hydrolysis	H^+		1.45	9
Sucrose inversion	H^+		2.1	10
Acetal hydrolysis	H^+		2.7	11
Acetal hydrolysis	H^{+*}		1.0*	11
Enolization of acetone†		H^+	2.1	12
Enolization of acetone†		HOAc	0.7	13
Glucose mutarotation		H^+	0.73	14
Glucose mutarotation		HOAc	0.39	14
Glucose mutarotation		H_2O	0.3	14

* Studied for a constant buffer ratio of HOAc to OAc^-.
† Followed by measuring rate of reaction with bromine.

$$v = \frac{k_s^H K_{HA}}{K_{SH^+}} \alpha C_S = k_\alpha^H C_S$$

where k_α^H is the first-order rate coefficient for the reaction with constant buffer ratio, $\alpha = C_{HA}/C_{A^-}$. A similar equation will hold for reaction in D_2O. Then, at constant α, the ratio of rate coefficients will be

$$\frac{k_\alpha^D}{k_\alpha^H} = \frac{k_s^D}{k_s^H} \frac{K_{SH^+}}{K_{SD^+}} \frac{K_{DA}}{K_{HA}} \tag{4}$$

The first two ratios in the term on the right are just as in Equation **3**, but the ratio K_{DA}/K_{HA} is new. This is for the ionization of the buffer acid *HA* in D_2O relative to H_2O. For typical weak acids such as acetic or benzoic, this ratio will be in the order of ⅓, and will approximately cancel the ratio K_{SH^+}/K_{SD^+}. Hence the observed rate ratio k_α^H/k_α^D for the constant buffer ratio case will be essentially equal to the term k_s^D/k_s^H, and will fall in the range 0.7 to 1.1. The fifth entry of TABLE 1 is an example of this for acetal hydrolysis. Comparison with the result for constant hydrogen ion concentration (fourth entry of TABLE 1) shows that the ratio drops for the constant buffer case by a factor of 2.7, roughly as predicted from a comparison of Equations **4** and **3**.

General acid catalysis with pre-equilibrium proton transfer. If a reaction characterized by pre-equilibrium formation of a conjugate acid is to exhibit general catalysis, the rate-determining step must involve a proton removal. The obvious further proviso is that the proton removed be different from the one that is added in the pre-equilibrium step. A well-known example is the acid-catalyzed enolization of a ketone, in which the proton is added in the first step to the carbonyl group, and the proton removed in the second slow step is from the alpha carbon of the ketone. To emphasize this point we shall call the substrate BSH, where H is the proton removed in the slow step and where B is the base site that is involved in conjugate acid formation. We can write the mechanism as

$$BS\mathrm{H} + \mathrm{H}X = \mathrm{H}BS\mathrm{H}^+ + X^- \qquad \text{(equilibrium)}$$

$$\mathrm{H}BS\mathrm{H}^+ + X^- \xrightarrow{\mathrm{k}_s} \text{Products} + \mathrm{H}X \qquad \text{(slow)}$$

We now note that, from the standpoint of comparison with experiment, this mechanism really encompasses four distinct situations. The predicted behavior differs when the catalyst is a strong acid ($\mathrm{H}X = \mathrm{H_3O^+}$) and when it is a weak acid ($\mathrm{H}X = \mathrm{H}A$). There are two additional limiting situations, depending on whether the proton that is removed in the slow step does or does not exchange with solvent at a rate that is large compared to the rate of the slow step itself. If exchange of the hydrogen to be removed is slow, we would have for the solvent D_2O

$$\mathrm{D}BS\mathrm{H} + X^- \xrightarrow{\mathrm{k}_s} \text{Products} + \mathrm{H}X, \qquad \text{(slow)}$$

but if this exchange is fast the slow step will be

$$\mathrm{D}XS\mathrm{D} + X^- \xrightarrow{k_s} \text{Products} + \mathrm{D}X, \qquad \text{(slow)}$$

The importance of this distinction lies in the fact that, when fast exchange occurs, the change from H_2O to D_2O as solvent causes both a *solvent* isotope effect and a *kinetic* isotope effect, whereas if the exchange is slow only the solvent isotope effect enters.

Let us consider first the case where exchange is negligible and catalysis is by a strong acid. If we here denote the rate coefficient for the slow step as $\mathrm{k}_s(\mathrm{H}^+)$, with $\mathrm{k}_s(\mathrm{D}^+)$ for solvent D_2O, we can write the rate as

$$v = \frac{\mathrm{k}_s(\mathrm{H}^+)}{\mathrm{K}_{\mathrm{H}XS\mathrm{H}^+}} C_{XS\mathrm{H}} C_{\mathrm{H_3O^+}} \qquad (5)$$

If we call the observed second-order rate coefficients $\mathrm{k^H}$ and $\mathrm{k^D}$, we can write for the relative rates

$$\left(\frac{\mathrm{k^D}}{\mathrm{k^H}}\right)_{\mathrm{H_3O^+}} = \frac{\mathrm{k}_s(\mathrm{D}^+)}{\mathrm{k}_s(\mathrm{H}^+)} \frac{\mathrm{K}_{\mathrm{H}BS\mathrm{H}^+}}{\mathrm{K}_{\mathrm{D}BS\mathrm{H}^+}} \qquad (6)$$

which is very similar to Equation **3** for specific hydrogen ion catalysis. We will again expect the ratio $\mathrm{k}_s(\mathrm{D}^+)/\mathrm{k}_s(\mathrm{H}^+)$ to be close to unity, and the ratio of ionization constants of the conjugate acids, $\mathrm{K}_{\mathrm{H}BS\mathrm{H}^+}/\mathrm{K}_{\mathrm{D}BS\mathrm{H}^+}$, should once more be in the order of from 2 to 4. Hence the predicted value of $\mathrm{k^D}/\mathrm{k^H}$

should fall in the rough range 1.5 to 3, very similar to that for specific catalysis by a strong acid. For the enolization of acetone, which is an example of this mechanism, the observed rate ratio is 2.1, in good agreement with this prediction.

If we still consider catalysis by a strong acid, but postulate rapid exchange of the proton to be removed, we will have for the rate ratio

$$\left(\frac{k^D}{k^H}\right)'_{H_3O^+} = \frac{k_s'(D^+)}{k_s'(H^+)}\,\frac{K_{HBSH^+}}{K_{DBSD^+}} \tag{7}$$

The ratio of ionization constants should again be in the order of from 2 to 4. However, the ratio of rate coefficients for the slow steps, $k_s'(D^+)/k_s(H^+)$, should be considerably less than unity, since a *kinetic* isotope effect is now additionally involved. The magnitude of this latter will depend on the chemical details of the slow step, but a reasonable range for $k_s'(D^+)/k_s(H^+)$ is from perhaps ⅓ to ⅕. Hence we now predict for the observed rate ratio k^D/k^H a ratio of less than unity, a reasonable estimate being in the order of from 1 to ½. As has been noted earlier,[15] an example of a reaction in which the proton removed exchanges rapidly with solvent is the mutarotation of glucose, and it is seen in TABLE 1 that for this case catalysis by a strong acid leads to $k^D/k^H = 0.73$, almost threefold smaller than the value for the acetone reaction. The implication is that the kinetic isotope effect for the glucose reaction is about 3, which is a quite reasonable value.

If the catalyst for this reaction class is a weak acid such as acetic, one can derive the general prediction that the ratio of second-order rate coefficients should be smaller than for the strong acid case by a factor of from 2 to 4.[5] Denoting the weak acid as HA and the rate coefficient for the slow step as $k_s(HA)$, we obtain the result

$$\left(\frac{k^D}{k^H}\right)_{HA} = \frac{k_s(DA)}{k_s(HA)}\,\frac{K_{HBSH^+}}{K_{DBSH^+}}\,\frac{K_{DA}}{K_{HA}} \tag{8}$$

Comparing this with the analogous Equation 6 for strong acid catalysis, we conclude that the ratio $(k^D/k^H)_{HA}$ will differ from that for the strong acid by a value of from ½ to ¼ (the value of the ratio K_{DA}/K_{HA}), *unless* the rate ratios for the slow steps in the two cases are very different. Since the only difference in the slow steps is that the attacking base is H_2O for the one situation and A^- for the other, it is reasonable to conclude that $k_s(D^+)/k_s(H^+) \approx k_s(DA)/k_s(HA)$. If this is true then the relation between the experimentally-observed rate ratios for the two catalysts should simply be

$$\frac{(k^D/k^H)_{HA}}{(k^D/k^H)_{H_3O^+}} \approx \frac{K_{DA}}{K_{HA}} \tag{9}$$

The observed data are in reasonable accord with this expectation. For the enolization of acetone the rate ratio for catalysis by strong acid is threefold larger than the ratio for catalysis by acetic acid. Recalling that the ratio of ionization constants of acetic and deuteroacetic acids is $K_{HOAc}/K_{DOAc} = 3.3$, this result is close to that predicted. A similar prediction should, of course, apply for reactions in which the proton to be removed in the slow step undergoes rapid exchange with the solvent. The results for the glucose mutarotation (TABLE 1) are in rough agreement with this prediction, in that the ratio for catalysis by the weak acid acetic is indeed smaller, by a factor of two, than the ratio for strong acid catalysis.

General acid catalysis with initial slow proton transfer. For catalysis by strong acids this mechanism is

$$S + H_3O^+ \xrightarrow{k_s(H^+)} \text{Products} + H_3O^+ \qquad \text{(slow)}$$

in which the essential point of the mechanism is that there are no pre-equilibrium proton transfers, so that the transition state is formed by direct interaction of substrate and acid. The experimental rate ratio is simply that for the slow step, $k_s(D^+)/k_s(H^+)$. The general expectation is that this ratio will be less than unity (normal solvent isotope effect) but, depending on the details of the bonding in the transition state, a modest reverse isotope effect is also possible.[5,16] Hence a reasonable prediction for this mechanism is that k^D/k^H will vary from perhaps 0.7 to 1.4, with less-than-unity values being more likely.

TABLE 2

PREDICTIONS OF RELATIVE RATES IN D_2O AND H_2O FOR MECHANISMS OF ACID-BASE CATALYSIS

Mechanism	k^D/k^H
Acid catalysis	
I. Specific hydrogen ion	1.4 to 3
II. General acid	
(a) Pre-equilibrium H transfer	
(1) Slow exchange of proton finally removed	
a. Strong acid catalyst	1.4 to 3
b. Weak acid catalyst	0.5 to 1
(2) Fast exchange of H removed	
a. Strong acid catalyst	0.5 to 1
b. Weak acid catalyst	0.2 to 0.35
(b) One-step slow proton removal	
a. Strong acid catalyst	0.7 to 1.4
b. Weak acid catalyst	0.2 to 0.35
(c) H-bonded complex	
Base catalysis	
I. Specific hydroxide ion	1.4 to 2
II. General base*	
(a) Pre-equilibrium proton transfer	0.15 to 0.25
(b) One-step slow H removal	
(1) Slow exchange of H removed	0.7 to 1.5
(2) Fast exchange	0.15 to 0.25

* It can be plausibly argued that the rates for general base catalysis will depend only slightly on base strength; hence this variable has not been explicitly considered.

There do not seem to be direct experimental data for this mechanism. The data of Gieb[17] on exchange of the aromatic hydrogens of resorcinol suggests a ratio value of close to unity, but interpretation is complicated by other effects. Similarly, Long and Watson[18] report a ratio of $k^D/k^H = 0.9$ for reaction of the enolate ion of methylacetylacetone with hydrogen ion, but the calculation of this value involves an assumption about relative acid strengths of the hydrogen and deutero enols.

For catalysis by a weak acid this reaction is

$$S + HA \xrightarrow{k_s(HA)} \text{Products} + HA \qquad \text{(slow)}$$

and the ratio of second-order rate coefficients is simply $k_s(DA)/k_s(HA)$. It is possible on the basis of rather general arguments to conclude that for this mechanism, just as for the pre-equilibrium mechanism for general acid catalysis, the rate ratio for catalysis by a weak acid will be several times smaller than the ratio for catalysis by a strong acid.[5] This conclusion is in agreement with the experimental data of Long and Watson, who found for the enolate ion mentioned above that the k^D/k^H ratio for catalysis by a molecular acetic acid was only 1/5 the magnitude of the same ratio for catalysis by hydrogen ion.

Still another possible mechanism for general acid catalysis is formation of a hydrogen-bonded complex.[4] Although this is occasionally postulated, there seem to be no well-established examples of it, even for the solvent water. Hence it does not seem important to make relative rate predictions for this case. In a sense this is fortunate, since the information on relative hydrogen bonding in D_2O is still rather incomplete.

Discussions very similar to the ones above can be given for the various mechanisms of base catalysis. The experimental data are less extensive in these cases, but it is possible to conclude that experiment and prediction are in reasonably good agreement.

TABLE 2 summarizes predictions for both acid and base catalysis. The figures in this table are in agreement with the general proposal that studies of relative rates in D_2O can be of assistance in determining mechanisms of acid-base catalysis. However, they also show that predictions for the various mechanisms overlap sufficiently, so that firm conclusions based on relative rate studies alone are only rarely possible.

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SOME ASPECTS OF DEUTERIUM ISOTOPE EFFECTS IN MECHANISM STUDIES

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During the course of several years' research with deuterium, tritium, and carbon-14, three major pitfalls or obstacles to the deduction of proper conclusions from isotopic data have been encountered or recognized. These are (1) the operation of the intermolecular isotope effect; (2) difficulty in distinguishing stereoselectivity from the intramolecular isotope effect; and (3) difficulty in distinguishing primary and secondary isotope effects.

The Intermolecular Isotope Effect

Although the theory of homocompetitive first-order reactions as applied to the intermolecular isotope effect has been known for some time,[1] the consequences of this theory are sometimes difficult to recognize in the laboratory. Fortunately, many experiments are possible in which deuterium can be employed "carrier-free," and this fact is one of the very great advantages of the use of deuterium over tritium. When either deuterium or tritium is employed in trace quantities, however, the homocompetitive nature of the reaction must be recognized.

$$A \xrightarrow{k} B$$

$$A^* \xrightarrow{k^*} B^*$$

If A and B are unlabeled reactant and product, A^* and B^* are labeled reactant and product, and k and k^* are the respective first-order specific rate constants, then n and n^* refer to the number of molecules or concentrations of A and A^* in a given reaction at time t, and n_0 and n_0^* represent these same molecules at time zero:

$$\frac{-dn(t)}{dt} = kn$$

$\frac{n}{n_0} = e^{-kt}$ = fraction of unlabeled molecules remaining at time t.

$f = 1 - e^{-kt}$ = fraction of unlabeled molecules that have undergone reaction at time t.

Similarly,

$f^* = 1 - e^{-k^*t}$ = fraction of labeled molecules that have undergone reaction at time t.

The ratio of product fraction of the labeled to unlabeled molecules at any time becomes:

$$R = \frac{1 - e^{-k^*t}}{1 - e^{-kt}}.$$

In a recent report[2] M. H. Lietzke and I published large-scale plots of R

versus f over a wide range of values of k^*/k (0.05 to 0.999), these plots having been obtained through the use of the ORACLE.† Given in FIGURE 1 are two typical curves for $(k^*/k) = 0.05$ and 0.30, respectively, for $f = 0.990 - 0.999$. These illustrate the "lagging" of the heavy isotope referred to by Melander,[16] and show that for sizable isotope effects, even after 99.9 per cent reaction of unlabeled reactant, a considerable fraction of labeled reactant remains.

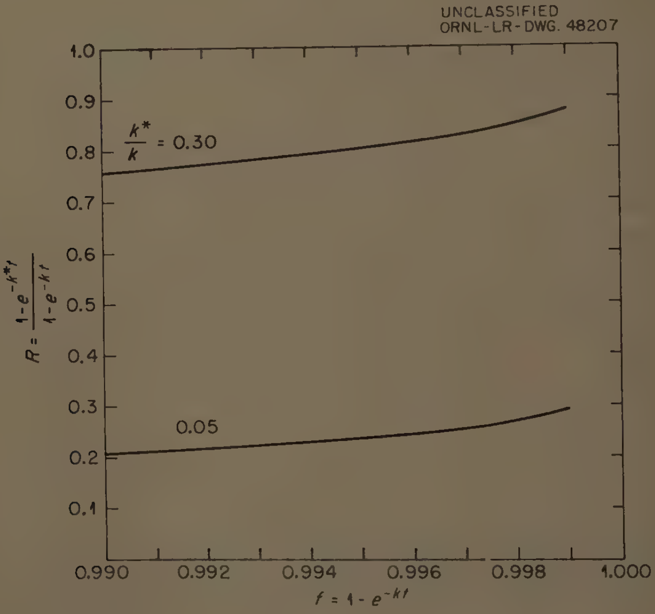

FIGURE 1.

In addition to the cumulative consequence of the isotope effect, illustrated in FIGURE 1, the differential plots have also been calculated.[1,2] At any time t the infinitesimal increment of the fraction of unlabeled product formed in the time interval dt for a first-order reaction is:

$$df = \frac{-dn}{n_0} = ke^{-kt}\,dt.$$

For the labeled product:

$$df^* = \frac{-dn^*}{n_0^*} = k^*e^{-k^*t}\,dt.$$

Then

$$x \equiv \frac{df^*}{df} = \frac{k^*}{k}\,e^{k(1-k^*/k)t},$$

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† Oak Ridge Automatic Computer and Logical Engine.

from which

$$1n\, x = \left(\frac{k^*}{k} - 1\right) 1n(1 - f) + 1n\,\frac{k^*}{k}.$$

The foregoing expression was derived by Downes.[1d] A typical differential curve (for $k^*/k \doteq 0.7$) is given in FIGURE 2, and illustrates the futility of attempting to draw conclusions from tracer experiments in which a sizable isotope effect is exhibited, and in which the isotopic ratio of product is determined for a low-yield reaction whose mechanism is unknown.

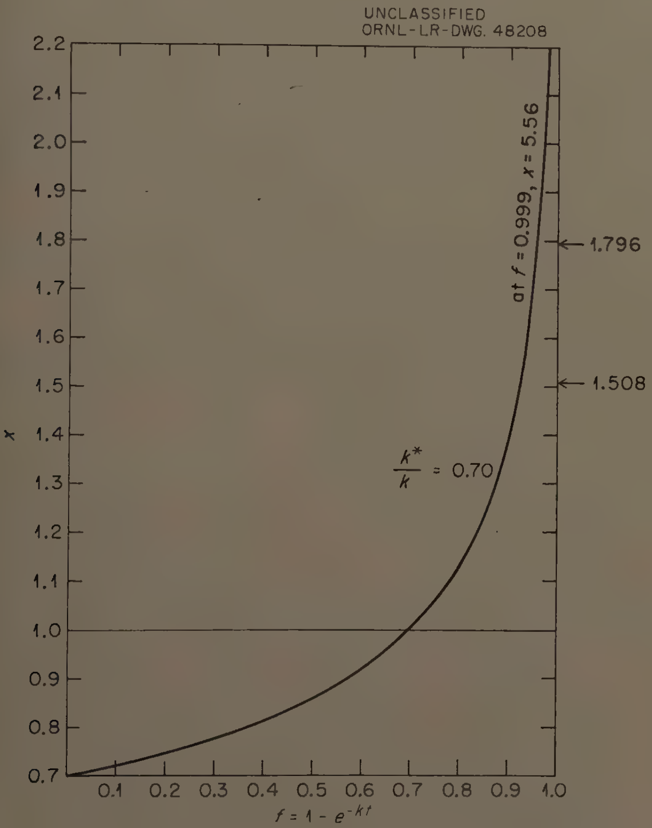
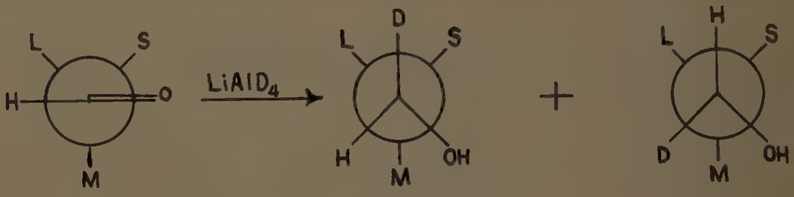


FIGURE 2.

Stereoselectivity and the Intramolecular Isotope Effect

The field of stereoselectivity, or stereospecificity in organic reactions, has received much attention in recent years.[3a,3b] For example, it has been shown that the addition of phenyl-magnesium bromide to optically active phenyl-labeled α-aminopropiophenone yields optically active 1,1-diphenyl-1-2-amino-1-propanol in which, within experimental error, only one of the two phenyls is labeled.[4] This stereoselectively labeled compound undergoes deamination in such a fashion that the labeled phenyl migrates to produce α-phenyl propiophenone of opposite sign of rotation than that exhibited by the α-phenylpropiophenone formed through migration of the unlabeled phenyl. In a similar fashion it is possible that the isotopes of hydrogen can exhibit stereospecificity. Such a situation could arise, for example, in the reduction of an aldehyde with lithium aluminum deuteride:

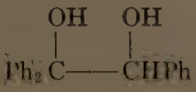


I II III

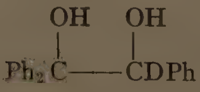
In the Formulas I, II, and III, L, M, and S refer to large, medium, and small groups. The point to be made here is that there is no a priori reason to assume that II and III should be formed in equal amounts. Thus in any attempt to determine an intramolecular isotope effect between hydrogen and deuterium on the mixture of II and III or derivatives thereof it must not be assumed without experimental evidence that the two positions occupied by hydrogen and deuterium are necessarily equivalent.

Difficulty in Distinguishing Primary and Secondary Isotope Effects

The primary isotope effect can be considered to occur during the slow or so-called "rate-determining" step of a reaction, with scission or formation of a bond to the heavier isotope. A secondary isotope effect can be exhibited, however, when the isotopic bond (for example, deuterium to carbon) is adjacent to the site of reaction, and is not broken during the rate-determining step. At first glance this problem might appear trivial. It was encountered, however, in our study of the hydrogen-deuterium isotope effect during the pinacol rearrangement of 1,1,2-triphenylethylene glycol (IV), 1,1,2-triphenylethylene-2-$^2H_1$ glycol (V), and certain derivatives of these substances.[5]


$$\begin{array}{c} \quad OH \quad OH \\ \quad | \qquad | \\ Ph_2C\text{---}CHPh \end{array}$$

IV


$$\begin{array}{c} \quad OH \quad OH \\ \quad | \qquad | \\ Ph_2C\text{---}CDPh \end{array}$$

V

Both radiochemical and kinetic data[5] indicated that, under a wide variety of conditions of acidity, both IV and V can undergo rearrangement with nearly quantitative loss of the tertiary hydroxyl group and with an isotope effect (k_H/k_D) of about 3. On first impression these data seemed to indicate that a secondary hydrogen-deuterium isotope effect had been exhibited. Further experiments, however, demonstrated[5] that, under the conditions studied kinetically, tertiary hydroxyl removal is extremely rapid and reversible, compared with the rate of product formation. Infrared and nuclear magnetic resonance spectra of the products showed that, during the reaction catalyzed by cold concentrated sulfuric acid, deuterium migration had taken place intramolecularly and quantitatively. Thus it was clear that hydrogen or deuterium migration to the tertiary carbonium center becomes rate determining. This problem has been considered also by Bunton,[6] Duncan,[7] Deno,[8] and Smith,[9] and their co-workers.

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HELIX-RANDOM COIL TRANSFORMATIONS IN DEUTERATED MACROMOLECULES*

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Introduction

Macromolecules such as proteins and nucleic acids are thought to contain internal hydrogen bonds, some of which are responsible for maintaining the specific folded configurations in which these molecules can exist. For example, in the α-helical configuration of proteins and polypeptides, each amide group is bonded to the third amide group along the backbone chain by $NH\cdots OC$ hydrogen bonds.[1] Since a protein can also exist in the randomly coiled form, in which it usually has lost whatever biological activity is possessed in the helical, or native, configuration, it is of great importance to understand the nature of the transformation from a helix to a random coil or, in other words, the relative stabilities of these two forms under a variety of environmental conditions. We shall, therefore, consider briefly some aspects of the equilibrium involved in the helix-random coil transition and some factors which can affect the equilibrium. Then we shall consider some experimental investigations of this equilibrium in which the substitution of deuterium for hydrogen has provided confirmation of current ideas about the role of hydrogen bonding in the internal structure of macromolecules. This problem assumes considerable importance since reversible transformations between helical and randomly coiled configurations may be a widely occurring phenomenon in biology. Therefore it is of interest to investigate the influence of deuterium-hydrogen substitution on the helix-random coil transition in model systems. The discussion here will be limited to proteins and synthetic polypeptides.

Acetic Acid Dimerization

In order to illustrate the difficulties involved in predicting the effect of deuteration on equilibria involving hydrogen bonds, it is worth while to begin with a consideration of a reaction involving the formation of a hydrogen bond in a low-molecular-weight model compound, for example, the dimerization of acetic acid in the gas phase. For this purpose it would be desirable to know the thermodynamic parameters for the reactions

$$2CH_3COOH \rightleftharpoons (CH_3COOH)_2 \qquad (1)$$

and

$$2CH_3COOD \rightleftharpoons (CH_3COOD)_2 \qquad (2)$$

Early data have been reported for the enthalpy of formation of the dimer,[2]

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according to Equations 1 and 2. However, the experimental error, ±700 cal. in the heats of association, is too large to detect the effect of deuteration. In the absence of direct thermodynamic data we may try to estimate the effect of deuterium-hydrogen substitution from vibrational assignments of Weltner[3] and Miyazawa and Pitzer.[4] Deuteration may be considered to change the zero-point energy for the various vibrational modes of the molecule. Consider for the moment only the O-H stretching frequencies ν obtained from infrared data which are listed in TABLE 1.

TABLE 1

O-H AND O-D STRETCHING FREQUENCIES FOR ACETIC ACID[3] IN CM.$^{-1}$

	CH_3COOH	CH_3COOD	CD_3COOH	CD_3COOD
Monomer	3546	2653	3640	2660
Dimer	2990	2260	3106	2299
$\Delta\nu$ (dimerization)	−556	−393	−534	−361

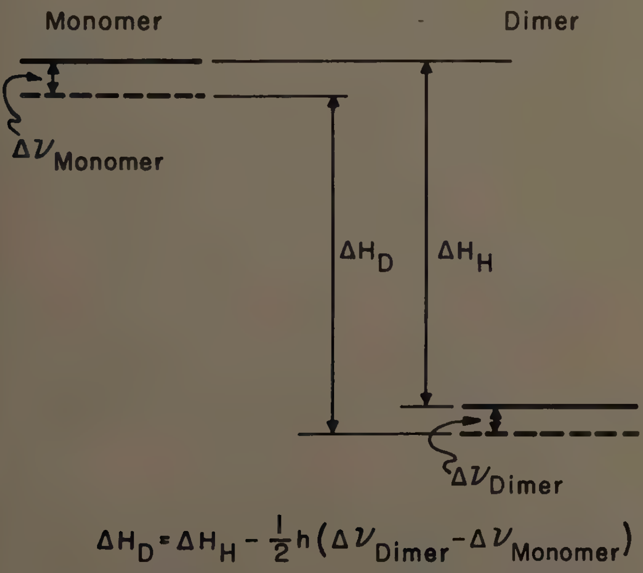

Monomer
Dimer
$\Delta\nu_{Monomer}$
ΔH_D
ΔH_H
$\Delta\nu_{Dimer}$
$\Delta H_D = \Delta H_H - \frac{1}{2}h(\Delta\nu_{Dimer} - \Delta\nu_{Monomer})$

FIGURE 1. Schematic representation of ground states for O-H (solid line) and O-D (dashed line) stretching frequencies in CH_3COOH and CH_3COOD.

The contribution of the zero-point energy to the heat of dimerization is $h\Delta\nu/2$ where h is Planck's constant. This quantity is $-278h$ for CH_3COOH and $-197h$ for CH_3COOD. Therefore, if the O-H stretching mode were the only one of concern, the deuterium dimer would be weaker by the difference in these two quantities, that is, by $81h$ or 231 cal. per dimer or 116 cal. per hydrogen bond (see also FIGURE 1). Of course, all the other frequencies, besides the O-H stretching, must be taken into consideration. While Halford,[5] Slutsky and Bauer,[6] and Miyazawa and Pitzer[4] have provided normal coordinate treatments of the vibrations, a complete and accurate assignment of all other pertinent frequencies for the hydrogen-containing and deuterated species is not available; therefore, their contribution to the zero-point energies cannot be properly evaluated. However, as a crude estimate, a consideration of the O-H bending frequencies[3] indicates that the 231 cal. can be increased or decreased by as much as 150 cal., depending on the vibrational mode selected. In addition, from estimates of the six low-frequency intermolecular modes of the dimer[4] (together with the *assumption* that the corresponding frequencies for the deuterated dimer are lower by a factor of $\sqrt{2}$), the 231 cal. should be decreased by about 300 cal., making the deuterium dimer stronger. Furthermore, since the intermolecular modes are of such low frequency,[4] a Boltzmann excitation factor would also have to be included, over and above the effect of the zero-point energies. The net effect is to indicate that it is difficult to predict which dimer is stronger until more accurate data are available for the various frequencies concerned. However, we may tentatively conclude from the above considerations that the deuterium dimer is stronger.

In the case of liquid water the hydrogen bonds in D_2O are slightly stronger[7] than those in H_2O. However, Pimentel and McClellan[8] point out that the ΔH data for carboxylic acid dimers are too fragmentary to provide support for the notion that the deuterium bond is stronger. The simple calculation indicated above supports the Pimentel-McClellan contention. The only other partly relevant available data are the vapor density measurements of Potter, Bender, and Ritter[9] for Reaction **1** above and **3** below.

$$2CD_3COOD \rightleftharpoons (CD_3COOD)_2 \tag{3}$$

The experimental heats of dimerization are −13.73 kcal./mole and −14.10 kcal./mole for Reactions **1** and **3**, respectively, indicating that the hydrogen bonds in the fully deuterated dimer are stronger. Consideration of the free energy of dimerization leads to the same conclusion, for example, at 80° C. the difference in the standard free energy of formation of the dimer for Reactions **1** and **3** is

$$\begin{aligned} \Delta F_D{}^\circ - \Delta F_H{}^\circ &= -170 \text{ cal. per dimer} \\ &= -85 \text{ cal. per hydrogen bond} \end{aligned} \tag{4}$$

indicating that the dimer of Reaction **3** is more stable than that of Reaction **1** at this temperature. If we try to apply the same simple calculation of the enthalpy changes to Reactions **1** and **3**, as we did to Reactions **1** and **2**, that is, attributing the difference to the effect of deuteration on the zero-point energies of the O-H stretching frequencies (TABLE 1), we find that the dimer of Reaction **1** should be stronger than that of Reaction **3** by 280 cal., which is opposite to the result indicated by experimental enthalpies of formation of Potter, Bender, and Ritter. The trouble, of course, lies again in the lack of knowledge of the pertinent frequencies and also in the fact that the CH_3 group in Reaction **3** is also deuterated, giving rise to a secondary isotope effect, the calculation of which would require accurate data and assignments of all the frequencies for the whole molecule. In light of this, we cannot conclude any more than that, in the absence of secondary isotope effects, the substitution of deuterium for hydrogen may possibly strengthen a hydrogen bond.

Helix-random Coil Transformation

The α-helix contains intramolecular NH···OC hydrogen bonds between peptide units. The transformation from the helix to the random coil involves the rupture of these bonds and a gain of configurational entropy. According to the above discussion of acetic acid, the substitution of deuterium for hydrogen might strengthen the NH···OC hydrogen bonds and affect the transformation from the helix to the random coil (in a hypothetical gas-phase reaction, or in the absence of specific solvent effects). It should be noted that the deuterated groups are sufficiently far apart so that there will probably be no secondary isotope effects involved.

The transformation from the helical to the randomly coiled form may be regarded as a phase transition which, under appropriate conditions, is reversible. Therefore, the transition temperature, T_{tr}, or melting point, may be considered as the temperature at which $\Delta F_{\mathrm{unf}} = 0$, where ΔF_{unf} is the free-energy change for unfolding an n-membered helix to the random coil. Following Schellman,[10] ΔF_{unf}, for a helix without side-chain groups, may be approximated by $n\Delta F_{\mathrm{res}}$ for sufficiently large n (that is, neglecting the effect of the chain ends), where ΔF_{res} is the free-energy change to unfold one amino acid residue in an infinitely long chain. While the effect of the side chains on ΔF_{unf} can be taken into account,[11] we shall neglect it here. Therefore, ΔF_{res} is also zero at T_{tr}, giving

$$T_{\mathrm{tr}} = \Delta H_{\mathrm{res}}/\Delta S_{\mathrm{res}} \tag{5}$$

where ΔH_{res} and ΔS_{res} are the enthalpy and entropy of unfolding one amino acid residue in an infinitely long chain. If deuterium is substituted for hydrogen, both ΔH_{res} and ΔS_{res}, and therefore T_{tr}, will be affected. Writing

$$\delta T_{\mathrm{tr}} = (T_{\mathrm{tr}})_{\mathrm{D}} - (T_{\mathrm{tr}})_{\mathrm{H}} \tag{6}$$

$$\delta H_{\mathrm{res}} = (\Delta H_{\mathrm{res}})_{\mathrm{D}} - (\Delta H_{\mathrm{res}})_{\mathrm{H}} \tag{7}$$

$$\delta S_{\mathrm{res}} = (\Delta S_{\mathrm{res}})_{\mathrm{D}} - (\Delta S_{\mathrm{res}})_{\mathrm{H}} \tag{8}$$

$$\delta F_{\mathrm{res}} = (\Delta F_{\mathrm{res}})_{\mathrm{D}} - (\Delta F_{\mathrm{res}})_{\mathrm{H}} \tag{9}$$

where the subscripts D and H refer to the situations when the molecule contains deuterium and hydrogen, respectively, the relative change in T_{tr} may be obtained from Equation 5 by differentiation.

$$\frac{\delta T_{\mathrm{tr}}}{T_{\mathrm{tr}}} = \frac{\delta H_{\mathrm{res}}}{\Delta H_{\mathrm{res}}} - \frac{\delta S_{\mathrm{res}}}{\Delta S_{\mathrm{res}}} \tag{10}$$

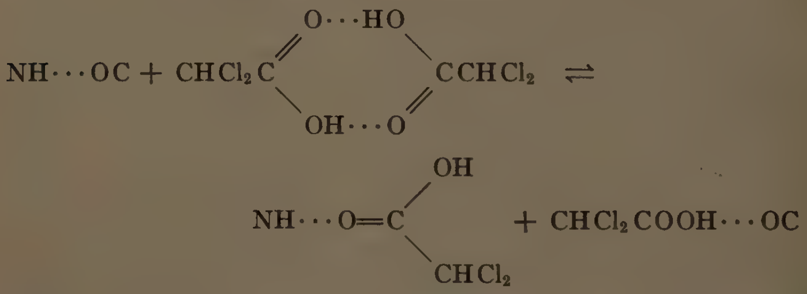
$$= \frac{\delta H_{\mathrm{res}}}{\Delta H_{\mathrm{res}}} - \frac{T_{\mathrm{tr}}\delta S_{\mathrm{res}}}{T_{\mathrm{tr}}\Delta S_{\mathrm{res}}} = \frac{\delta F_{\mathrm{res}}}{\Delta H_{\mathrm{res}}} \tag{11}$$

since $\Delta H_{\mathrm{res}} = T_{\mathrm{tr}}\Delta S_{\mathrm{res}}$. If the deuterium bond were stronger than the hydrogen bond, δF_{res} would be positive. Thus, since ΔH_{res} is positive, T_{tr} would increase when deuterium is substituted for hydrogen. The magnitude of the increase would depend on the numerical values of these parameters. If δF_{res} were about +100 cal. and ΔH_{res} about 7 kcal., then $\delta T_{\mathrm{tr}}/T_{\mathrm{tr}}$ would be +0.014. If T_{tr} were about 300° K., then δT_{tr} would be about +4°. Such a small change in T_{tr} can easily be detected by determining the fraction of the molecules which is unfolded at a given temperature, taking T_{tr} as the temperature at which one half the molecules are unfolded. In practice such data are directly obtainable from measurements of optical rotation as a function of temperature. A plot of specific rotation at a given wave length, $[\alpha]_D$, for example for, the sodium D line, against temperature gives the temperature dependence of the fraction unfolded.

Before we try to examine experimental data to see the effect of deuterium-hydrogen substitution on T_{tr}, we must first recognize that proteins are dissolved in liquid solvents which themselves have the possibility of hydrogen bonding to the NH and CO groups. For example in water the helix-random coil transformation would have to be written as

$$\mathrm{NH\cdots OC + HOH\cdots OH_2 \rightleftharpoons NH\cdots OH_2 + HOH\cdots OC} \qquad (12)$$

The thermodynamics of this reaction have been considered in some detail by Schellman.[10] In a nonaqueous solvent containing a species such as dichloroacetic acid, the helix-random coil transformation would have to be written as



$$\mathrm{NH\cdots OC + CHCl_2C\begin{matrix} \diagup\!\!\diagup O\cdots HO \diagdown \\ \diagdown OH\cdots O \diagup\!\!\diagup \end{matrix}CCHCl_2 \rightleftharpoons NH\cdots O{=}C\begin{matrix} \diagup OH \\ \diagdown CHCl_2 \end{matrix} + CHCl_2COOH\cdots OC} \qquad (13)$$

For some proteins in water at room temperature, for example, ribonuclease,[12] the helix is the stable form. Upon heating to elevated temperatures the helix is transformed into the hydrated random coil. In the case of the synthetic polypeptide poly-γ-benzyl-L-glutamate (PBG) dissolved in a 20:80 (v/v) mixture of dichloroethane and dichloroacetic acid, the random coil is stable at room temperature.[13] The thermodynamic parameters of Reaction **13** are such that heating to elevated temperatures leads to a transformation from the random coil to the helix,[13] that is, a transformation in the reverse sense from that experienced in ribonuclease. Presumably, the helix would revert to a random coil if heated to still higher temperatures.

According to the above discussion of acetic acid, the substitution of deuterium for hydrogen would affect the hydrogen bonds of all the species of Reactions **12** and **13**. The over-all effect of deuterium on the equilibrium constants of Reactions **12** and **13** cannot be predicted on an a priori basis since we cannot make a sufficiently accurate prediction of the strengths of the various hydrogen bonds involved, and of the effect of deuterium thereon, as in the case of the acetic acid dimer. We must therefore rely on experiment to demonstrate the effect of deuterium-hydrogen substitution on these equilibria. If the hydrogen bonds of the various species have different strengths, then we should observe a shift in the equilibrium constant or transition temperature, but we cannot predict even the direction of the shift. However, the experimental demonstration of such a shift would add confirmation to current hypotheses that internal hydrogen bonds are an important structural feature of proteins and synthetic polypeptides. It might also provide a basis for understanding some of the biological effects of D_2O.

Poly-γ-benzyl-L-glutamate[14]

Using the optical rotation method mentioned above, the transition temperature was determined for hydrogen-containing and deuterium-containing samples of PBG (mol. wt. = 1–2 × 10^5) in a 20:80 (v/v) mixture of dichloroethane and dichloroacetic acid. Deuterated dichloroacetic acid was used with deuterated PBG. The results[14] are shown in FIGURE 2. It may be seen that the higher (positive) specific rotations at elevated temperatures indicate that the helix is the stable form at the higher temperatures.[13] Furthermore, the transition temperatures are 29° C. and 41° C. for the deuterated and hydrogen-containing polymers, respectively, demonstrating a very significant alteration in the strengths of the various hydrogen bonds upon deuteration.

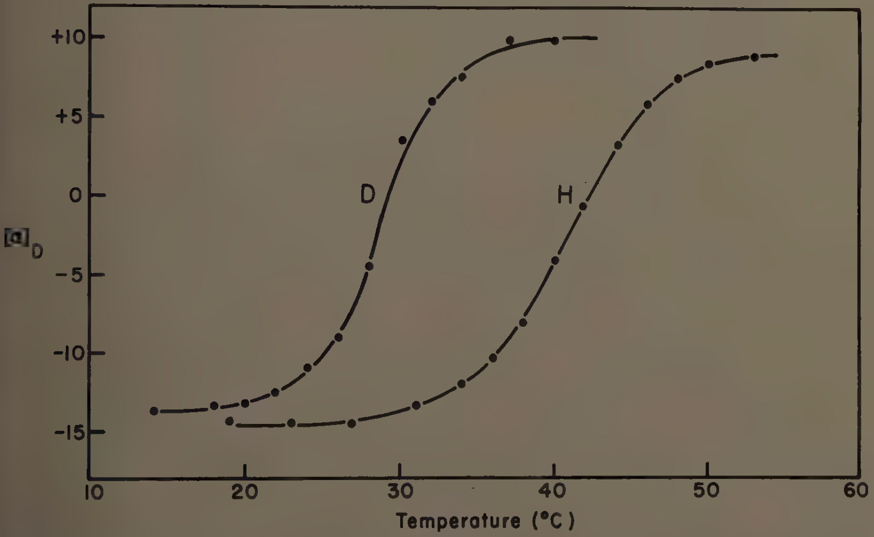


FIGURE 2. Specific rotation (Na_D line) as a function of temperature for hydrogen-containing (*H*) and deuterated (*D*) solutions of PBG in a 20:80 (v/v) mixture of dichloroethane and dichloroacetic acid.[14] A large $[\alpha]_D$ corresponds to a high ratio (helix)/(random coil). Reproduced by permission of the *Journal of the American Chemical Society.*[14]

It is of interest to note from FIGURE 2 that, at a temperature of about 35° C., the fraction of molecules in the helical form is very small for the hydrogen-containing sample and very large for the deuterated sample, showing the very large effect of deuterium substitution in a small temperature range. It is thus very interesting to speculate that the ratio of the concentrations of helices to random coils in a biological system (for example, in genes) can be markedly affected by deuteration. The effect of deuteration on the helix/random coil ratio also has implications for the interpretation of data from experiments on the kinetics of deuterium-hydrogen exchange.[15] In the latter technique, it is assumed that the substitution of deuterium for hydrogen does not change the helix/random coil ratio. However, if such a study were carried out at a temperature near the transition region (for example 35° C. in the case considered here), deuteration would affect this ratio. Of course if the kinetics experiment is carried out at a temperature sufficiently removed from the transition region, then this ratio will presumably not be affected. It seems probable that most of the experiments heretofore carried out on the kinetics of deuterium-hydrogen exchange have not been performed near the transition region. The effects of D-H substitution on the stability of helical structures in proteins is indicated by the example of ribonuclease discussed in the next section.

Ribonuclease[16]

There is a variety of experimental evidence which indicates that ribonuclease exists in a folded form at room temperature and unfolds as the temperature is raised[12,16,17] (C. Tanford and R. E. Weber, private communication; J. Hermans, Jr. and H. A. Scheraga, unpublished results), the transition temperature being *p*H dependent (C. Tanford and R. E. Weber, private communication; J. Hermans, Jr. and H. A. Scheraga, unpublished results). The effect of D-H substitution on the transition temperature at a given *p*H was therefore investigated[16] by the same optical rotation method used for PBG. The results are shown in FIGURE 3, where curves H and D represent the specific rotation of ribonuclease solutions in H_2O and D_2O, respectively. The higher (negative) specific rotations at elevated temperatures are compatible with the conversion of a helical to a randomly-coiled form at high temperatures. The transition temperatures are 61.9° and 66.1° C. in H_2O and in D_2O, respectively, again demonstrating an alteration in the strengths of the various hydrogen bonds upon deuteration.

The heat of transition, ΔH, which can be obtained[12,16] (C. Tanford and R. E. Weber, private communication) from curves H and D, is about +90 kcal./mole, the same within experimental error in H_2O and in D_2O. If an equation of the type of Equation **11** is applied to this result then $\delta F = +1$ kcal./mole, where δF is the change in ΔF_{unf} when hydrogen is replaced by deuterium. Since there are a minimum of 20 hydrogen bonds which are ruptured in the transition,[17] then the maximum value of δF_{res} is +50 cal./mole *in aqueous solution*, which is a reasonable value, considering the various hydrogen bonds involved in Reaction **12**. These results provide evidence that hydrogen bonds stabilize the structures of ribonuclease and PBG in solution. In conjunction with other evidence, it has provided a basis to postulate a three-dimensional model of ribonuclease.[18]

Summary

There are not yet sufficient data available, even for low-molecular-weight model compounds, to predict the effect of deuteration on the equilibria involving the formation of hydrogen bonds. Nevertheless the nature of the effects to be expected are known. Specifically, in the absence of secondary isotope effects, the substitution of deuterium for hydrogen might possibly strengthen a hydrogen bond. However, since a variety of different types of hydrogen bonds are involved in equilibria between helical and randomly coiled macromolecules in solution, the effect of deuteration on these equilibria cannot be predicted. From experiments it is apparent that deuteration leads to a measurable effect on the helix-random coil transition temperature for a particular protein and synthetic polypeptide. This effect provides evidence for the presence of internal hydrogen bonds in these macromolecules, and may also provide a clue as to the mode of action of D_2O in biological systems. In the particular examples cited, $\Delta H < 0$ for PBG (Reaction **13**), and deuteration decreases T_{tr}; $\Delta H > 0$ for ribonuclease (Reaction **12**) and deuteration increases T_{tr}. Thus δT_{tr} has the same sign as ΔH as a result of deuteration, implying that $\delta F > 0$ for these two systems. Also, in both systems, deuteration favors the helical form. Other examples will have to be considered before it can be determined whether this is a general property of hydrogen-bonded macromolecules.

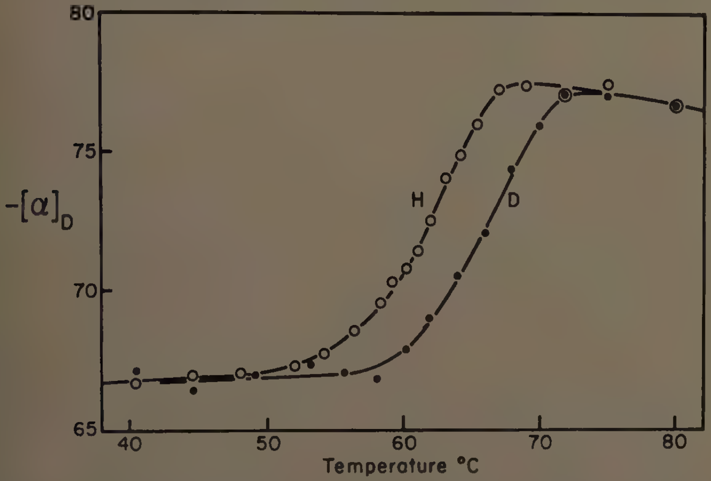


FIGURE 3. Specific rotation (Na$_D$ line) as a function of temperature for hydrogen-containing ribonuclease in H_2O (*H*) and deuterated ribonuclease in D_2O (*D*).[16] For comparison, Curve *D* has been shifted vertically downward by 2 units. It is possible, however, that the difference in $[\alpha]_D$ between H-containing ribonuclease is real. Reproduced by permission of the Elsevier Publishing Company, Amsterdam, Holland.[16]

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CHLOROPLAST PIGMENTS AND PHOTOSYNTHESIS IN DEUTERATED GREEN ALGAE*

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Introduction

The discovery[1a,b] that certain algae can live and grow in very high concentrations of heavy water presents a large number of research possibilities. In this communication we describe the isolation and characterization of deuterio-† chlorophylls *a* and *b* from the chloroplasts of fully deuterated green algae, together with some preliminary observations on photosynthesis in deuterated algae.

The algae used in this work were cultured for more than a year in a nutrient solution prepared from 99.8 per cent D_2O. There is every reason, therefore, to expect that the compounds biosynthesized by the algae will be essentially fully deuterated, since all of the hydrogen requirements of the autotrophic algae are satisfied by water. Nevertheless, the possibility that the algae select hydrogen in preference to deuterium from the small amount of H_2O present in 99.8 per cent D_2O, while remote, is not entirely excluded. The infrared studies reported here provide independent evidence that the chlorophyll made by the algae is essentially free of hydrogen. This evidence, together with the results of similar studies on the algal carotenes and carbohydrates, shows that the organisms are indeed fully deuterated.

Perhaps the most significant difference between the metabolism of hydrogen and deuterium compounds in organisms is the difference in their reaction rates arising from the kinetic isotope effect. The deuterated organisms may have essentially the same set of chemical reactions, but these will proceed at different absolute and relative rates compared to those of the ordinary hydrogen compounds. Comparative studies may thus contribute to the unravelling of the complex kinetics characteristic of metabolism. Deuterated algae may, therefore, have a particularly useful role in the study of photosynthesis.

Extraction and Properties of Deuterio-Chlorophylls a and b

Fully deuterated algae for preparation of deuterio-chlorophylls a and b. The algae used for the extraction of the chloroplast pigments were *Chlorella vulgaris* and principally *Scenedesmus obliquus*. The algae had been maintained in 99.8 per cent D_2O media for more than a year; the details of the mass culturing of these organisms are described elsewhere in this monograph (H. L. Crespi *et al.*). The cultures were uni-algal but not necessarily sterile. Freshly harvested organisms were generally employed for extraction of the deuterated pigments. Ordinary chlorophylls were extracted for comparison purposes from cultures of *C. vulgaris* grown in ordinary water, from clover or spinach.

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* The work described in this paper is based on work performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

† The prefix deuterio- is used in this paper to indicate the fully deuterated compound.

Extraction and Chromatography of the Chlorophylls

The algae, harvested by centrifugation, were treated for one minute with boiling water and then rapidly cooled to room temperature. Unless the cells were heated in this way extraction of the pigments with organic solvents was very slow and incomplete. We have found short heating to be preferable to long heating that may alter the chlorophylls. The deuterated algae were treated with boiling D_2O to minimize exchange, but ordinary water was used in subsequent manipulations. The heat-treated organisms were once more collected by centrifugation and the pigments extracted with a mixture of 2 volumes of absolute methanol and 1 volume of petroleum ether (b.p. 20 to 40° C.). Ordinary methanol, CH_3OH was used. Since the sugar columns subsequently used contain exchangeable hydrogen, nothing is gained by using CH_3OD at this point. The extraction of the algae was repeated until the residual cells were almost colorless; three extractions were generally sufficient for complete extraction. The crude methanol-petroleum ether extracts were combined in a separatory funnel and washed several times with aqueous sodium chloride until all of the methanol had been removed from the petroleum ether layer. The separated aqueous methanol layer was re-extracted with petroleum ether, and the petroleum ether extracts were combined.

The ratio of deuterio-chlorophyll *a* to *b* in crude extracts of the deuterated algae was determined by the method of Comar.[2] The ratio of deuterio-chlorophyll *a* to *b* in deuterated *S. obliquus* was in the range of 1.5:1 to 2:1. The deuterated algae thus appear to have slightly more chlorophyll *a* relative to *b* than the ratio previously reported for ordinary green algae,[3] but the slightly enhanced chlorophyll-*a* content is of doubtful significance.

The chloroplast pigments extracted from the algae were separated by column chromatography on confectioners' powdered sugar containing 3 per cent starch, and the chromatograms were developed with 0.5 per cent *n*-propanol in petroleum ether. The chlorophyll *a* was washed through the column; the elutriate was washed with water to remove the propanol, and the solvent was evaporated under reduced pressure. The chlorophyll-*b* zone was removed from the column. The pigment was eluted with petroleum ether and ethanol, the solution was washed with water, and then evaporated under reduced pressure. The first samples of deuterated chlorophyll obtained by this procedure showed very strong C—H stretching absorptions in the infrared as well as C—D absorption. The crude petroleum ether extract of deuterated algae when examined after removal of the petroleum ether showed practically no C—H absorption in the infrared. This indicated that hydrogen-containing impurities were introduced from the sugar column. This inference was confirmed by experiment. When a sugar column was treated with 0.5 per cent *n*-propanol-petroleum ether and the solvent evaporated a residue was obtained. This residue smelled like burnt sugar and exhibited very intense carbon-hydrogen and carbonyl absorptions in the infrared. We estimate it to be present to the extent of about 1 part in 15,000. Most of the contaminant can be removed from the sugar by prewashing the column with an ethanol-petroleum ether mixture. If care is taken to remove the residual alcohol by copious washing with petroleum ether alone, the adsorption characteristics of the column are not adversely affected. Colorless compounds present in the algae may also be incompletely separable from the chlorophyll pigments by chromatography, and consequently the chlorophylls were further purified by a number of separations from petroleum ether plus alcohol by removal of the alcohol.

Improved procedure for the isolation and purification of the chlorophylls. Because the chlorophylls isolated by the usual chromatographic procedure followed by evaporation of the elutriate may be contaminated by certain plant constituents and by organic substances from the sugar columns, and because it is often difficult or impossible to induce small quantities of the resolved pigments to separate from petroleum ether, we have developed a dependable procedure that overcomes all these difficulties. As other workers may be confronted with similar purification problems we now describe this method as applied to spinach, a rich source of chlorophyll that is usually available at all seasons. Quantities of plant material, volumes of solvent, and size of chromatographic columns may be varied in proportion.

Fresh spinach leaves free of midribs (200 gm.) were dropped into vigorously boiling water (about 2 l.). After one to two minutes the water was cooled quickly with an excess of cold water. The water was then decanted, the leaves being held back with a brush. The leaves were washed once with cold water and squeezed by hand, then between paper towels in the hand. These water-extracted leaves, which weighed from 95 to 100 gm., were separated from one another and placed in methanol (500 ml.) plus petroleum ether (125 ml. b.p. 20 to 40° C.). After a few minutes most of the chlorophyll was removed. The deep-green extract was decanted through a soft pad of cotton into a large separatory funnel (3 l.). The leaves were re-extracted twice with the same quantities of methanol and petroleum ether that were used to make the first extraction. Water (250 ml.) was then added to the combined extracts, and the solution was swirled gently. If an emulsion was formed, or if the petroleum ether layer was very small, a small amount of concentrated sodium chloride solution was added. The lower methanol layer was then drawn into another separatory funnel and shaken vigorously with petroleum ether (60 ml.). Sometimes addition of a little more sodium chloride solution was required. The yellow-green methanol layer, which was about 80 per cent methanol and which contained much of the strongly adsorbed, minor xanthophylls, was separated and discarded. The petroleum ether layer was added to the principal petroleum ether solution that was then mixed with methanol (200 ml.) and water (50 ml.). The aqueous methanol was separated and extracted with petroleum ether (60 ml.) which was also added to the principal petroleum ether solution. The latter was again re-extracted with methanol (200 ml.) plus water (50 ml.). After separation from the petroleum ether layer, the methanol was extracted with petroleum ether (60 ml.) which was combined with the principal petroleum ether solution. To prevent separation of the pigments, diethyl ether (60 ml.), from a freshly opened can, was added to the petroleum ether, from which the residual alcohol was removed by several extractions with water (200 ml. each).

The petroleum ether was added to a chromatographic column of powdered sugar (3 by 12 inches) prepared by pressing small portions of the sugar into the chromatographic tube.[4] The sugar (3 lb.) was the untreated, commercial product. Percolation of the solution through the sugar was accelerated with slightly reduced pressure (about 45 to 56 cm. of mercury). By the time all the solution had been added to the column, the chlorophylls formed a zone 2 to 3 inches deep. The solvent containing the nonsorbed carotene then reached nearly to the bottom of the column. At this stage, the column was washed with fresh petroleum ether (about 100 ml.) and then with petroleum ether containing 0.5 per cent *n*-propanol. The nonsorbed carotene was carried into the percolate and collected separately. The chlorophyll *a* was carried into the percolate next, and it too was collected separately. The lutein plus zeaxanthin, the quantity of which had been reduced by the methanol extractions of the petroleum ether, was washed into the percolate and discarded. At this stage, the chlorophyll *b* was near the bottom of the column. The sugar above the chlorophyll-*b* zone, with very small quantities of the residual minor xanthophylls, was removed and discarded, whereupon the chlorophyll *b* was washed from the column with petroleum ether containing ethanol (3 to 5 per cent).

Each chlorophyll was then isolated in a solid state. To this end, the petroleum ether solutions were placed in separatory funnels and shaken or swirled gently with water several times to remove the residual alcohol. Then the petroleum ether was shaken vigorously with water (150 ml.), the chlorophyll collecting on the surface of the water droplets. This mixture was permitted to stand for a few minutes for the petroleum ether to rise, any clear water layer was discarded, and the water plus chlorophyll layer was drained into another separatory funnel. This extraction procedure was repeated until the petroleum ether layer was nearly colorless. Sometimes as many as 8 to 10 extractions were required. When each aqueous layer was drained into the second separatory funnel, much of the water separated and could be drained away. Finally there was obtained a little petroleum ether with suspended solid chlorophyll and a little dispersed water. All this material was drained into a flask, the chlorophyll on the walls of the funnel being loosened with a fine stream of water, and the mixture was cooled with solid carbon dioxide. After several hours, the petroleum ether plus chlorophyll was decanted from the frozen water, and the chlorophyll was collected by centrifugation. If much solid chlorophyll remained with the ice, the latter was melted, and more petroleum ether (about 25 ml.) was added. This was shaken; the water was frozen again; and the petroleum ether plus solid chlorophyll was poured off and centrifuged. The chlorophyll collected by centrifugation was dried in a vacuum desiccator. From 200 gm. of fresh spinach the yields of the separated chlorophylls were: *a*, 0.12 to 0.15 gm., and *b*, 0.05 to 0.06 gm.

Each chlorophyll, prepared as just described, was purified further by chromatography, by additional partition between methanol and petroleum ether, and by precipitation. As an example, chlorophyll *a* (0.25 gm.) was dissolved in fresh diethyl ether (50 ml.) which was then diluted with petroleum ether (200 ml.). This solution was adsorbed in a column of powdered sugar (3 by 12 inches with 3 lb. of sugar). The adsorbed chlorophyll *a*, which formed a zone about 1.5 inches deep, was then washed with petroleum ether plus 0.5 per cent *n*-propanol. Traces of pheophytin *a* and chlorophyll *a′* were carried along ahead of the chlorophyll *a*. When the chlorophyll-*a* zone reached the bottom of the column, the sugar above it was removed, and the pigment was eluted with petroleum ether plus 2 to 3 per cent ethanol, thus providing a solution of chlorophyll *a* in about 300 ml. of petroleum ether. This petroleum ether solution was treated with methanol (200 ml.) and then with water (50 ml.) and the aqueous methanol was separated. This treatment was repeated. Then the petroleum ether was washed several times with water by gentle swirling and cooled overnight with solid carbon dioxide. The solid chlorophyll that separated was collected by centrifugation and dried in vacuum. It weighed about 0.23 gm.

Chlorophyll *b* was purified by the same procedure, except that the column was washed with petroleum ether containing from 1 to 1.5 per cent *n*-propanol.

Spectral absorption curves of the purified chlorophylls agreed well with those reported before.[5]

The deuterio-chlorophylls were prepared from the algal extracts by this same procedure. They behaved like the normal hydrogen-containing chlorophylls in every respect.

Absorption spectra in the visible region. The effect of replacement of hydrogen by deuterium in chlorophyll on the visible absorption spectra has been carefully studied. To the extent that the electronic transitions are affected by the vibrations of the molecule, the replacement of hydrogen by deuterium may be expected to produce small but observable changes in the visible absorption spectrum. Preliminary measurements[6] failed to reveal these changes unequivocally, but more careful measurements now show small but real differences in the visible absorption spectra of ordinary and deuterated chlorophylls.

The absorption spectra in the visible region were usually determined with a Cary Model 14 recording spectrophotometer. To minimize instrumental errors, two different instruments were used, and the results were confirmed with a Beckman DK-1 recording instrument. Three principal properties were determined, the absorption curves, the wave lengths of the absorption maxima, and the difference spectra. These difference spectra were measured with a solution of ordinary chlorophyll in one light beam and a solution of the deuterio-chlorophyll in the other, concentration and light path being adjusted so that absorption at most wave lengths was equal.

The wave lengths of the absorption maxima of ordinary and deuterated chlorophyll *a* and *b* are given in TABLES 1 and 2, the spectra for chlorophyll *a* in FIGURE 1. The maxima of the deuterated chlorophylls occur at slightly shorter wave lengths than those of the maxima of the ordinary pigments. This difference between the maxima of the chlorophylls *a* in methanol solution is about 10 Å. For chlorophyll *b*, dissolved in a variety of solvents, the difference is less, of the order of 5 Å, and is apparently independent of the solvent as shown by TABLE 2. There is no evident change in the peak-height ratio for the corresponding ordinary and deuterated chlorophylls. A similar study was made of allomerized chlorophyll *a* and *b*, with results as shown in TABLE 4. Allomerization was carried out by allowing chlorophyll solutions in methanol to stand exposed to air for three days. Like the ordinary chlorophylls, the deuterio-chlorophyll *a* and *b* underwent the allomerization reaction with a shift of the absorption maxima. The difference between the wave lengths of the spectral maxima for allomerized *a* and allomerized deuterio-*a* was of the same magnitude as that between *a* itself and deuterio-*a*. A similar difference was also observed with the allomerized *b* compounds.

The visible absorption spectra establish the identity of the green pigments from the deuterated algae as chlorophyll *a* and *b*. Although spectral differences are detectable, nevertheless they are so small as to render unlikely the possibility that the ordinary and deuterio-chlorophylls differ in structure.

TABLE 1
ABSORPTION MAXIMA OF ORDINARY AND DEUTERIO-CHLOROPHYLL *A* IN METHANOL AND DIETHYL ETHER
Absorption Maxima (Å)

Solvent	Ordinary *a*		Deuterio-*a*	
	Red	Blue	Red	Blue
Methanol	6661–6667	4320–4330	6650–6656	4310–4330
Diethyl ether	6604–6607	4284–4288	6596–6600	4275–4280

TABLE 2
ABSORPTION MAXIMA OF ORDINARY* AND DEUTERIO-CHLOROPHYLL *B* IN VARIOUS SOLVENTS†
Absorption Maxima (Å)

Solvent	Ordinary *b*		Deuterio- *b*	
	Red	Blue	Red	Blue
Methanol	6520–6530	4695–4705	6515–6525	4690–4700
Diethyl ether‡	6415–6420	4515–4525	6405–6415	4510–4520
N,N-dimethyl formamide	6470–6480	4595–4600	6465–6475	4585–4595
Trifluoroethanol	6495–6505	4675–4685	6490–6500	4670–4680

* From clover.
† Eastman "Spectro" grade.
‡ From a freshly opened container.

Infrared spectra of ordinary and deuterio-chlorophyll a and b. While changes in the visible absorption spectra arising from the replacement of hydrogen by deuterium in chlorophyll are small, the situation is very different in the infrared region. The replacement of hydrogen by deuterium makes a major change in the vibrational spectrum. The infrared spectra of ordinary and deuterio-chlorophyll have therefore been intensively studied. Measurements were made with a Beckman IR-4 infrared spectrometer, using micro equipment. Infrared spectra were measured both in solution and on solid films. For the solution spectra, carbon tetrachloride was employed as the solvent. Concentrated solutions were necessary, and these were obtained by dissolving about 3 mg. of chlorophyll in about 10 μl. of carbon tetrachloride. The solution was then transferred to a 100 μ microcell by means of a hypodermic needle and syringe. To obtain the chlorophyll spectrum without the solvent absorption

2 mg. of chlorophyll were placed between two KBr plates that were heated on a melting point block to 150° C. for about a minute. The chlorophyll softened like wax and was distributed between the plates by application of slight pressure. These spectra were much superior to those obtained with conventionally prepared KBr pressed disks, in which there was considerable light scattering. The spectra obtained from solutions and from films on KBr plates[6] agreed very well, but for detailed work, the infrared spectra obtained in solutions are preferable because the peaks are better defined. The spectra obtained from films of chlorophyll are useful, however, in exploring regions in which carbon tetrachloride absorbs strongly.

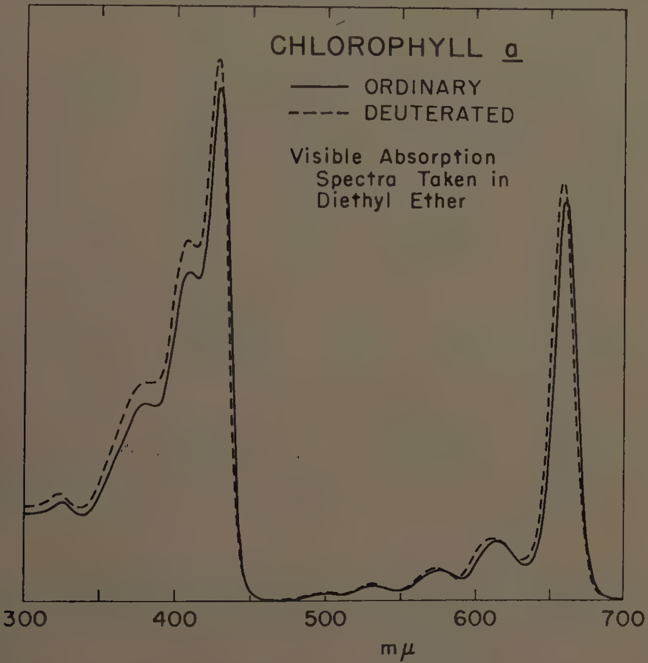


FIGURE 1. Absorption spectra of ordinary and deuterio-chlorophyll *a* in diethyl ether. Optical density, log (I_0/I) versus wave length.

Infrared spectra of ordinary and deuterio-chlorophylls *a*, in solution, and *b*, on plates, are presented in FIGURES 2 and 3. (After submission of the original manuscript of this paper, and with increased experience, spectral curves of the deuterio-chlorophyll *b* were obtained with much smaller CH peaks than those shown in FIGURE 3.) The important absorption peaks are listed in TABLE 4.

The infrared absorption spectra of chlorophyll have been measured by Holt and Jacobs,[7a] Stoll and Weidemann,[7b] and Weigl and Livingston.[8] Our measurements on ordinary chlorophyll are in general agreement with the earlier ones. Neither a detailed analysis of the infrared spectra nor a detailed comparison of ordinary and deuterated chlorophyll will be given here. However, a number of conclusions can be drawn from even a cursory examination of the spectra.

The most striking feature of the infrared spectra is the replacement of the prominent C—H absorption at 2900 cm.$^{-1}$ arising from C—H stretching vibra-

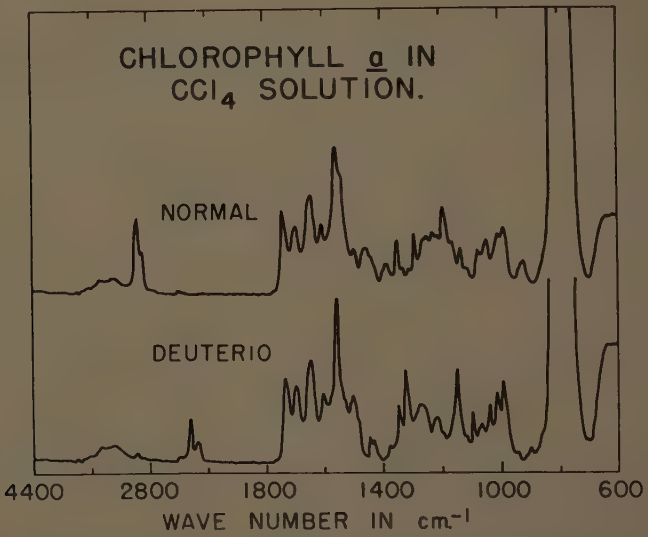


FIGURE 2. Infrared spectra of ordinary and deuterio-chlorophyll *a* in carbon tetrachloride solution (absorption maxima of CCl_4: 780, 980, 1007, 1218, 1258, 1553 cm.$^{-1}$).

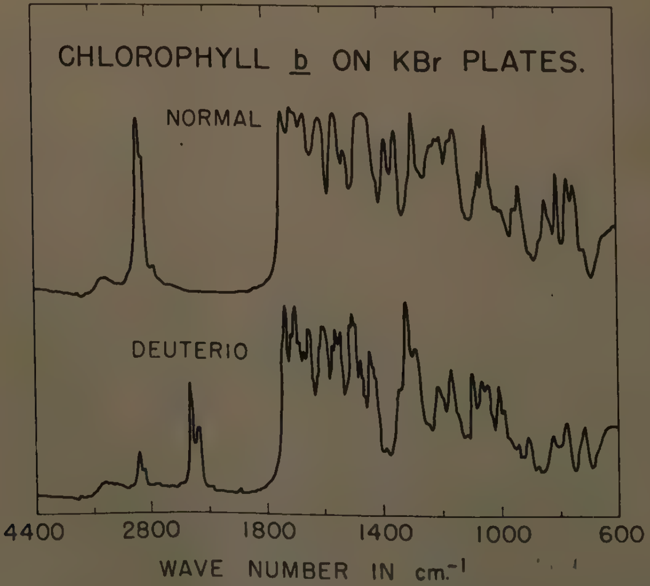


FIGURE 3. Infrared spectra of ordinary and deuterio-chlorophyll *b* taken on KBr plates.

tions in the ordinary chlorophylls, by a C—D absorption at 2150 to 2250 cm.$^{-1}$ in the deuterio-chlorophylls. This provides strong evidence that the green pigments are essentially fully deuterated when the algae are grown in heavy water. In the spectra of deuterio-chlorophyll *a*, very little if any absorption due to C—H can be detected. Thus far it has not been possible to obtain deuterated chlorophyll *b* spectra completely free of C—H absorption. Since it is difficult to believe that entirely different biosynthetic paths are employed for the synthesis of chlorophyll *a* and *b*, we prefer to attribute the C—H ab-

TABLE 3

ABSORPTION MAXIMA OF ALLOMERIZED ORDINARY AND DEUTERIO-CHLOROPHYLL *A* AND *B* IN METHANOL SOLUTION

Absorption Maxima (Å)

	Ordinary		Deuterated	
	Red	Blue	Red	Blue
Allomerized chlorophyll *a*	6580–6590	4200–4210	6565–6575	4185–4200
Allomerized chlorophyll *b*	6370–6380	4575–4585	6360–6370	4570–4580

TABLE 4

IMPORTANT INFRARED ABSORPTION MAXIMA OF ORDINARY AND DEUTERIO-CHLOROPHYLL *A* AND *B*

(cm.$^{-1}$)*

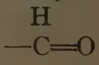
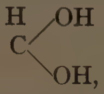
Chlorophyll *a*		Chlorophyll *b*	
Ordinary	Deuterio	Ordinary†	Deuterio‡
	1034		1036
1042		1043	
	1088		1087
1131	1142	1147	1157
1192			
1288		1290	
	1316		1311
1346	1341	1349	
1386		1382	
	1437		1437
1495	1498		1499
1602	1599	1606	1598
1647	1645	1664	1649
1692	1690	1702	1700
1734	1730	1735	1732
	2130–50		2150
	2245		2250
2875		2880	
2935		2935	

* All wave lengths have been corrected for instrumental variation.

† In addition to the peaks given in the table, a considerable number of other peaks can be observed. Thus, for ordinary chlorophyll *b* the following additional peaks have been observed in films on KBr plates: 713, 742, 764, 801, 840, 923, 1524, and 1553 cm.$^{-1}$

‡ Additional peaks observed in films of deuterated-chlorophyll *b*: 708, 771, 812, 863, 889, 930, 981, and 999 cm.$^{-1}$

sorption present in the spectra of deuterio-chlorophyll *b* to small amounts of hydrogen-containing impurities derived from the solvents and powdered sugar chromatographic columns. Our conclusions in this respect are reinforced by the fact that beta-carotene[6] and other chloroplast pigments, alpha-carotene and lutein, can be obtained from deuterated algae free of significant C—H absorption (H. H. Strain *et al.*, unpublished work). The intensity of the C—D stretching absorptions, in agreement with other observations on deuterium compounds, is distinctly weaker than are corresponding C—H stretching vibrations.

From TABLE 4 it appears that the set of 4 peaks in the 1600 to 1735 cm.$^{-1}$ region are at the same frequencies in both ordinary and deuterio-chlorophyll *a* and *b*. These four peaks then do not involve hydrogenic vibrations. If these absorption peaks do in fact arise from identical vibrations in both chlorophyll *a* and *b*, and do not involve hydrogen, then there is no prominent peak characteristic of the aldehyde carbonyl in the infrared spectrum of chlorophyll *b*. Holt and Jacobs dealt with this situation by ascribing one of the four peaks in the carbonyl region to an aldehyde carbonyl absorption, but this then required an *ad hoc* explanation for the appearance of a peak at very similar frequency in the chlorophyll *a* spectrum, where no aldehyde absorption can be expected. Weigl and Livingston, on the other hand, did not assign any absorption peak to the aldehyde group. We may assume, alternatively, that the aldehyde peak cannot be seen, because the aldehyde group exists as a hydrate,  $-\overset{\mathrm{H}}{\mathrm{C}}{=}\mathrm{O}$ versus  $\overset{\mathrm{H}}{\mathrm{C}}\begin{matrix}\mathrm{OH}\\ \mathrm{OH,}\end{matrix}$ as in the case of chloral hydrate. Such a supposition is consistent with the tenacity with which water is retained by chlorophyll, and with the presence of OH absorption in the infrared spectra of chlorophyll *b*. However, there may also be another explanation for the absence of a distinctive aldehyde absorption band in the infrared spectrum of chlorophyll *b*. The absorption by the aldehyde group in *b* may not be sufficient to appear as a separate peak above those due to the phytyl, pyrrole, vinyl, ester, and keto groups common to the ordinary and deuterio-*a* and *b*.

A detailed analysis of the infrared spectra will be facilitated by measurement of the infrared spectra of ordinary and deuterated phytol. The infrared spectrum of ordinary phytol was measured by Weigl and Livingston, but their material was impure as it absorbed strongly in the 1700 cm.$^{-1}$ region. We have measured the infrared spectrum of phytol on a sample that shows no absorption peaks in this region (carbonyl). It exhibited the same spectrum after purification by chromatography on magnesia. The principal infrared absorption maxima occur at 752–6, 1006, 1168, 1246, 1381, 1464, 1666, 2875, 2930 and the OH absorption at 3345 cm.$^{-1}$. As larger amounts of deuterated chlorophyll become available an effort to prepare fully deuterated phytol will be made.

Chromatographic comparison of chlorophyll a and deuterio-chlorophyll a. A mixture of chlorophyll *a* and the deuterio-*a* was sorbed in a column of powdered sugar and washed with petroleum ether containing 0.5 per cent *n*-propanol.

No separation of the pigment zone was observed. However, infrared measurements of the chlorophyll from the lower half of the green zone indicated a small increase in proportion of the ordinary *a*.

Formation of deuterio-chlorophyll a′ and deuterio-chlorophyll b′. When solutions of the deuterio-chlorophylls in *n*-propanol were heated to 100° C. for 1 hour and then chromatographed in a column of powdered sugar, as described by Strain and Manning[9a,b] for isomerization of the ordinary chlorophylls, each deuterio-chlorophyll yielded a similar less sorbed pigment. Each of these reaction products exhibited spectral absorption properties like those of the parent deuterio-chlorophylls. Like the parent chlorophylls, these products yielded a positive yellow, phase test when treated with strong sodium hydroxide in methanol. The product from deuterio-chlorophyll *a* was partially reconverted into deuterio-*a* when its solution in *n*-propanol was heated for 1 hour, and the product from deuterio-*b* was partially reconverted to deuterio-*b*. By analogy with the common chlorophylls, these products of the heat treatment are deuterio-chlorophylls *a*′ and *b*′.

When treated with hydrochloric acid, the deuterio-chlorophylls *a* and *b* were converted into the respective pheophytins. These substances, the deuterio-pheophytins *a* and *b*, were readily separable from the parent deuterio-chlorophylls in columns of powdered sugar. The deuterio-pheophytin *a* was less sorbed than the deuterio-pheophytin *b*.

With acids the deuterio-chlorophyll *a*′ and the deuterio-chlorophyll *b*′ were converted into the respective, magnesium-free deuterio-pheophytin *a*′ and deuterio-pheophytin *b*′. When a mixture of the deuterio-pheophytins *a* and *a*′ was chromatographed in a column of powdered sugar, two poorly separated zones were obtained, but when a mixture of ordinary pheophytin *a* and deuterio-pheophytin *a* were chromatographed, only one pigment zone was observed. Similarly, a mixture of the hydrogen-containing pheophytin *a*′ and the deuterio-pheophytin *a*′ were inseparable in the sugar columns. From these observations the deuterio-chlorophylls undergo isomerization reactions like those exhibited by the common chlorophylls. The deuterio-chlorophylls and their isomerization and acid-alteration products are separable from one another in columns of powdered sugar, but the deuterio-compounds are not separable from the corresponding hydrogen-containing compounds by chromatography in columns of sugar.

Photosynthesis in Deuterated Algae

The ready availability of fully deuterated algae (H. L. Crespi *et al.*, elsewhere in this monograph) presents a unique opportunity for the study of photosynthesis. The replacement of hydrogen by deuterium should affect both the absolute and relative rates of the reactions involved in the conversion of carbon dioxide to organic compounds without preventing the photosynthetic reactions. Some preliminary studies along these lines are reported here.

Preparation of algae. Scenedesmus obliquus and *Chlorella vulgaris* were grown in both ordinary and deuterium media (H. L. Crespi *et al.*, elsewhere in these pages) at 25 to 27° C. on a 4 per cent CO_2-mixture in nitrogen. The algae were illuminated continuously by overhead fluorescent lighting at an intensity of 700 ft.-ca. Algae were harvested by centrifugation at full speed in an International clinical centrifuge. The cells were washed once with water or deuterium oxide; no washing injury of the type described by Clendenning *et al.*[10] appeared to result. The cells were then resuspended in the appropriate solution and the packed cell volume determined after centrifugation for 20 min. For all these studies, a 1 per cent suspension of algae in buffer solution was made by suspending 0.5 ml. of packed cells in 50 ml. of buffer solution. It was important to use algae from fresh thriving cultures, as older algal cultures were much less active in photosynthesis. The effect of starving the algae by keeping them in the dark while supplied with air was examined and found not to affect the rate of uptake of carbon dioxide significantly.

Photosynthesis flask. A specially constructed flask was used for carbon dioxide uptake studies. A ground-glass, standard-taper, 125-ml. Erlenmeyer flask (male joint) was fitted with a cap (female joint) having a tapered opening at the top, extending for about 2 cm., over which was affixed a rubber injection-type stopper. This permitted the removal of an aliquot of the solution by hypodermic syringe and precluded loss of carbon dioxide from the system. Experiments specifically designed to check this point showed that no measureable diffusion of CO_2 through the rubber cap occurred during the experiments. The cap was held tightly to the flask by springs fixed to glass prongs. A circular glass shelf, about 1 cm. in diameter and having a lip 4 mm. high, was fitted to the inner wall of the flask just above the 50-ml. mark.

$NaHC^{14}O_3$ stock solution. A stock solution of C-14 labeled $NaHCO_3$ was prepared by dissolving 2 mc. of the isotope in 4 ml. of *p*H 9 phosphate buffer solution. The solution was assayed by diluting a 25-λ aliquot to the 50-ml. mark of a volumetric flask. A 100-λ aliquot of this dilution was added to 15 ml. of liquid scintillator in a 5-dram vial and the system was counted in a Tri-Carb liquid scintillation spectrometer (Model 314). A count rate of about 30,000 cpm/100 λ was observed.

Procedure. In the photosynthetic studies involving the effect of *p*H, 50 mg. of $NaHCO_3$ and 25 λ of $NaHC^{14}O_3$ solution (12.5 μc.) were placed on the shelf of the special flask. The algal suspension, prepared as described above, was added to the flask, below the shelf, with the aid of a long stemmed funnel. The flask was capped and, at zero time, was shaken vigorously to distribute the $NaHCO_3$ and isotope throughout the algal suspension. The flask was then placed on the mechanical shaker where the algae were permitted to photosynthesize. At 90-min. intervals for a total of six hours a small aliquot was removed from the flask by hypodermic syringe, centrifuged for 3 min., and 100 λ of the supernatant solution was counted.

Calibration curves. At *p*H 7 and below, a significant portion of the $NaHCO_3$ is present as CO_2, part of which is in solution and part in the gas phase. In order to deduce the amount of CO_2 plus bicarbonate present in the photosynthesis vessel from a measurement of the C^{14} content of an aliquot of the liquid phase, a calibration curve was necessary. (This can actually be achieved by calculation, but some of the necessary constants are not available for the deuterium system. An empirical calibration curve supplied a more certain answer.) The calibration curve was prepared by dissolving various amounts of C^{14}-labeled $NaHCO_3$ in buffer at a given *p*H, and then measuring the C^{14} content of an aliquot taken from the liquid phase. In this way a curve was prepared for each buffer solution relating mg. of $NaHCO_3$ equivalent in the gas phase versus mg. of $NaHCO_3$ in the liquid phase. A similar set of curves was prepared for the deuterium oxide buffers. For the phosphate buffers the calibration curves were straight lines, but for the *tris* (tris[hydroxymethyl]-aminomethane) buffers in the region *p*H 7 to 8, there were deviations from a straight line. More CO_2 was bound in the liquid phase than expected, due probably to the formation of carbamate. Since the calibration curves we have prepared apply only to the vessels and conditions we have used, the curves are not given here. At *p*H values above 8, the amount of CO_2 in the gas phase is negligible, and a direct count of the liquid phase is all that is required.

Buffers. In making a comparison between uptake of bicarbonate by ordinary and deuterated algae it is essential to determine the effect of change in *p*H on the rate of bicarbonate uptake. The electrochemistry of H_2O and D_2O may be very different, and it is important to distinguish between kinetic effects due to replacement of hydrogen by deuterium and rate changes that may occur in systems very sensitive to changes in *p*H. Solutions in H_2O and D_2O made up to be stoichiometrically identical will have markedly different H^+ and D^+ concentrations and activities. We have therefore examined the rate of photosynthesis in both ordinary and deuterated algae as a function of *p*H. It appears that in the range *p*H 6 to 8 the rate is insensitive to *p*H, and consequently the rate differences between ordinary and deuterated algae are attributable to metabolic rate effects involving deuterium rather than to *p*H or concentration effects.

For *p*H 7 and below a phosphate buffer system (KH_2PO_4-$NaOH$) was used. For *p*H 7 and above a tris(hydroxymethyl)aminomethane-HCl buffer system was employed. The deuterium buffers were prepared from KD_2PO_4-$NaOD$ and the tris buffer from tris(hydroxymethyl)aminomethane (in which all of the exchangeable hydrogen was replaced by deuterium) and DCl. Buffer solutions were made to *p*H of 6.0, 7.0, 8.0, 8.5, and 9.0. The corresponding buffers were made up in D_2O using the same stoichiometry as for H_2O, and the "*p*H" measured with a glass electrode. By making an appropriate correction[11,12] to the *p*H-meter reading it was established that the buffer solutions in D_2O corresponding to the H_2O buffer values mentioned above had *p*D values of 6.3, 7.3, 8.3, 8.8, and 9.4.

Carbon dioxide uptake studies. Carbon dioxide (or bicarbonate) uptake was studied over a six-hour period. The algae were agitated continuously at 25 to 27° C. at a light intensity of 700 ft.-ca. supplied by fluorescent lamps. The *p*H range was varied over the range *p*H 6.0 to 9.0, and comparisons were made between algae grown in H_2O and D_2O.

For both ordinary and deuterated algae the rate of CO_2 uptake was fairly constant in the range *p*H 6 to 8. The absolute amount of $NaHCO_3$ taken up appears to be independent of the initial amount added. Above *p*H 8.5 the rate is markedly depressed, and at *p*H 9 the algae are damaged severely in a short time. At all *p*H values the CO_2 uptake is greater in H_2O than in D_2O, as shown in FIGURES 4, 5, 6, and 7. In general the amount of C^{14} taken up in

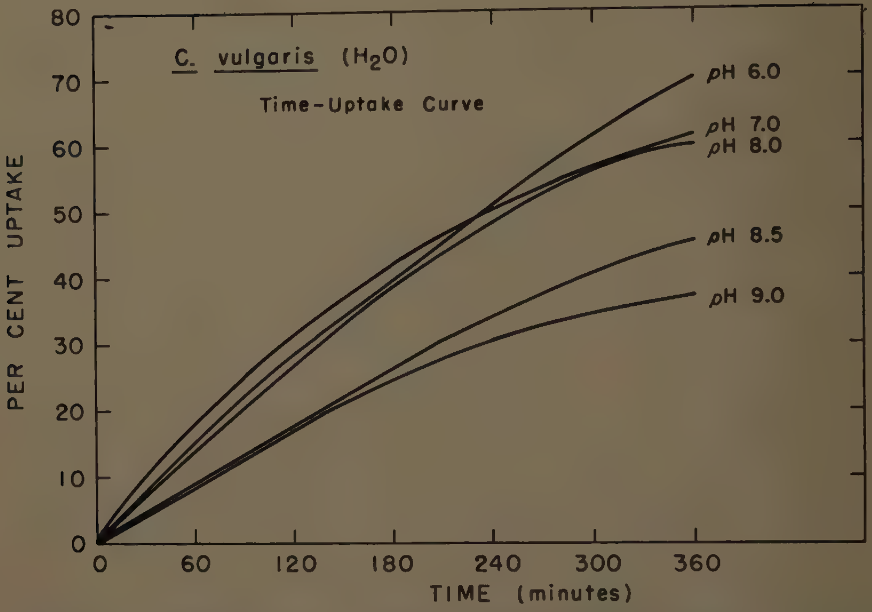


FIGURE 4. Uptake of $NaHC^{14}O_3$ by ordinary *C. vulgaris* in H_2O as a function of *p*H and time.

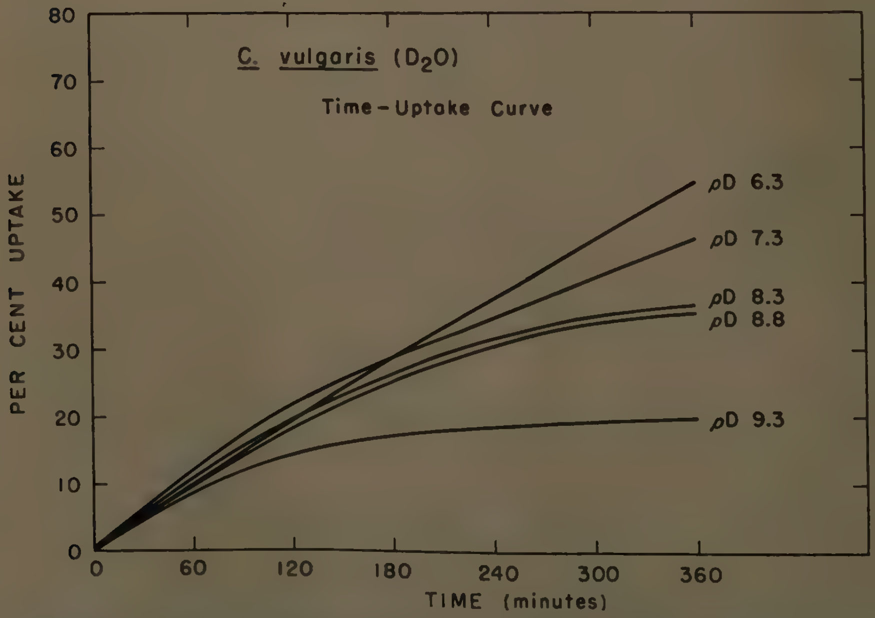


FIGURE 5. Uptake of $NaHC^{14}O_3$ by deuterated *C. vulgaris* in D_2O as a function of *p*D and time.

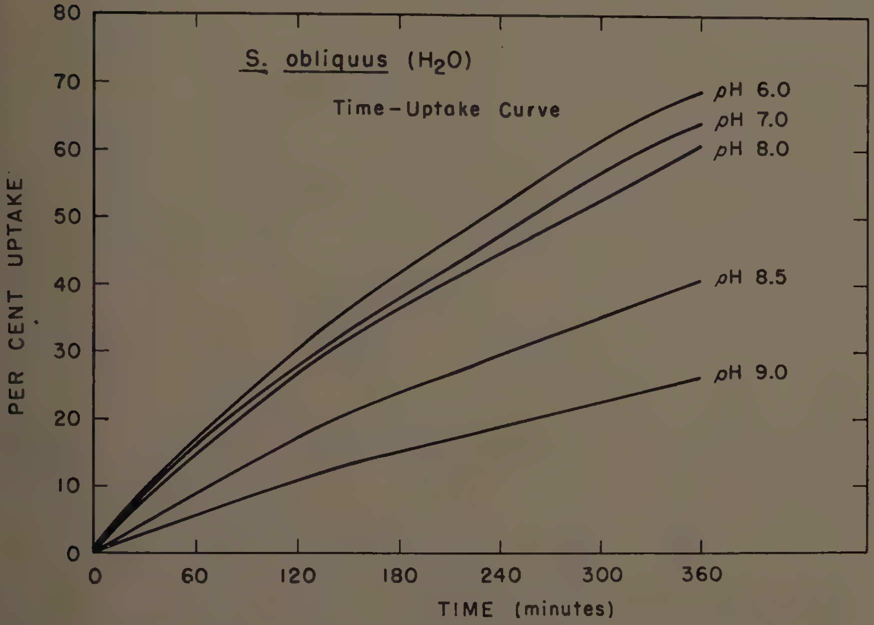


FIGURE 6. Uptake of $NaHC^{14}O_3$ by ordinary *S. obliquus* in H_2O as a function of *p*H and time.

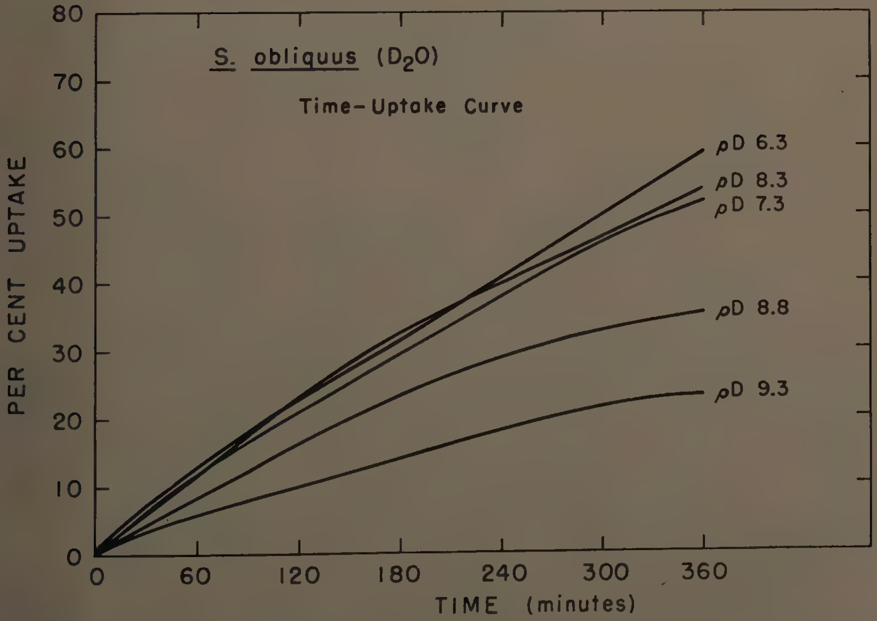


FIGURE 7. Uptake of $NaHC^{14}O_3$ by deuterated *S. obliquus* in D_2O as a function of *p*D and time.

D_2O by the deuterated algae was 10 to 30 per cent less than for ordinary algae in H_2O under the experimental conditions employed here. In some respects the surprising thing is that the differences in the rate of photosynthesis are not larger. It is hoped to extend these results by examining the effect of light intensity and temperature on the relative rates of photosynthesis of ordinary and deuterated algae.

Tolbert and Zill[13] have reported that at *p*H values greater than 4.5 *Chlorella* excretes as much as 10 per cent of the total $C^{14}O_2$ fixed as glycolic acid. Some experiments were therefore conducted to determine whether significant amounts of glycolic acid (or other organic C^{14}-labeled compounds) were excreted into the medium under our conditions. Concentrated hydrochloric acid was added to samples of the media used for photosynthesis and the solution flushed with nitrogen. In no case were any residual C^{14} counts detected sufficiently above background to confirm the presence of significant amounts of labeled C^{14} metabolic products in the solution. Under the conditions used in our experiments, particularly with macroscopic rather than essentially weightless amounts of $NaHCO_3$, no significant amount of glycolic acid is excreted into the medium.

TABLE 5

COMPARISON OF PHOTOSYNTHESIS OF ORDINARY *CHLORELLA VULGARIS* IN H_2O AND D_2O

	*p*H 7			*p*H 8		
Time (min.)	Uptake in H_2O (%)	Uptake in D_2O (%)	Ratio % Uptake D_2O/ % Uptake H_2O	Uptake in H_2O (%)	Uptake in D_2O (%)	Ratio % Uptake in D_2O/ % Uptake in H_2O
90	22.0	9.7	0.43	17.1	11.0	0.64
180	38.4	19.6	0.51	34.4	18.8	0.55
270	48.4	28.6	0.59	48.4	28.4	0.59
360	60.0	37.8	0.63	57.8	37.6	0.65

TABLE 6

COMPARISON OF PHOTOSYNTHESIS OF DEUTERATED *CHLORELLA VULGARIS* IN H_2O AND D_2O

	*p*H 7			*p*H 8		
Time (min.)	Uptake in H_2O (%)	Uptake in D_2O (%)	Ratio % Uptake D_2O/ % Uptake H_2O	Uptake in H_2O (%)	Uptake in D_2O (%)	Ratio % Uptake in D_2O/ % Uptake in H_2O
90	18.8	16.8	0.89	16.6	15.6	0.94
180	27.4	27.6	1.01	23.4	24.4	1.04
270	32.8	36.2	1.10	30.0	34.4	1.13
360	37.6	44.0	1.17	36.6	44.0	1.20

Reverse CO_2 uptake studies. Many years ago Craig and Trelease[14] measured the rate of photosynthesis of ordinary *Chlorella* in D_2O and observed a marked depression in the rate of photosynthesis. It is now possible to examine the reverse situation by introducing deuterated algae into H_2O, and some preliminary experiments along these lines have now been carried out.

The uptake of $NaHC^{14}O_3$ by ordinary and deuterated *C. vulgaris* in H_2O and D_2O has now been measured. These experiments were carried out by the techniques and under the experimental conditions used for the photosynthesis experiments described above. The results obtained when ordinary *C. vulgaris* is allowed to undergo photosynthesis in H_2O and D_2O are shown in TABLE 5. It is clear that the uptake of $NaHC^{14}O_3$ is markedly depressed in D_2O as compared to H_2O at both *p*H 7 and 8. The results of the reverse experiment, in which the behavior of deuterated *C. vulgaris* is examined in H_2O and D_2O is given in TABLE 6. It would appear that for the deuterated algae there is no significant difference in the rate of fixation of $NaHC^{14}O_3$ in H_2O or D_2O. For the hydrogen organism, it makes a considerable difference, under the conditions employed in these experiments, whether they carry out photosynthesis in H_2O or D_2O. For the deuterated organisms, however, it appears to make very little difference whether the medium for photosynthesis is H_2O or D_2O. This would imply that a reaction involving water is rate-determining for the ordinary hydrogen algae, but that it is not a rate-limiting step for the deuterated algae under the conditions of light and temperature used here.

These preliminary experiments must be extended to determine the effects of variation in light intensity and temperature before more specific conclusions can be drawn. Nevertheless, it is not unreasonable to expect that the availability of fully deuterated algae may make a significant contribution to the study of photosynthesis.

Acknowledgment

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CYTOCHEMICAL STUDIES ON DEUTERATED GREEN ALGAE*

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The production of essentially fully deuterated cultures of the green algae, *Scenedesmus obliquus* and *Chlorella vulgaris*, is described elsewhere in this monograph (H. L. Crespi *et al.*). The differences in growth pattern of deuterated cultures as compared with ordinary (protium) cultures grown in H_2O suggests dissimilarities between individual cells of ordinary† and deuterated organisms. In an effort to elucidate some of these differences with special reference to the localization of specific substances in cells, cytochemical studies have been conducted on deuterated algae and on parallel hydrogen organisms.

Morphology

Deuteration results in alteration of the mean cell dimensions in both *S. obliquus* and *C. vulgaris*. This was determined from size measurements made with an ocular micrometer on cells chosen at random from cultures in all stages of their life cycle. Measurements of *S. obliquus* were made at the widest and the longest parts of the cell. Under the culture conditions that were employed,[1] *S. obliquus* has a mean width of $4.16 \pm 0.030\ \mu$ and a mean length of $6.46 \pm 0.056\ \mu$. Corresponding deuterated cells have a mean width of $3.91 \pm 0.044\ \mu$ and a mean length of $7.99 \pm 0.050\ \mu$. Thus the average deuterated *S. obliquus* is longer and thinner than the average ordinary hydrogen *S. obliquus*. Deuterated *C. vulgaris* is spheroid as is its ordinary prototype. However, while the ordinary cells have a mean diameter of $5.14 \pm 0.041\ \mu$, the deuterated cells are substantially larger with a mean diameter of $5.42 \pm 0.033\ \mu$. Although the mean sizes of fully deuterated and ordinary hydrogen algae differ from each other, this difference, as may be seen in FIGURES 1 and 2, results not from the exclusion of any cell size type, but rather from alteration in the frequency distribution of the various cell size types. The alteration caused by deuteration in *S. obliquus*' morphology is especially interesting. It resembles the alteration caused in this species by incubation at reduced temperature.[1] The long, thin cell form so common in the deuterated cells occurs in ordinary hydrogen cells as they are newly arisen following division. However, ordinary hydrogen cells soon become shorter and thicker. Deuterated cells tend to retain their juvenile form.

The difference between the morphology of deuterated and ordinary cells is not confined to external form. The nucleus of both *S. obliquus* and *C. vulgaris* is larger in the deuterated than in the hydrogen cells. In *C. vulgaris* some cells

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* This paper is based on work performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

† In this paper, organisms grown in H_2O are referred to as ordinary or hydrogen organisms, and organisms grown in D_2O are referred to as deuterated organisms.

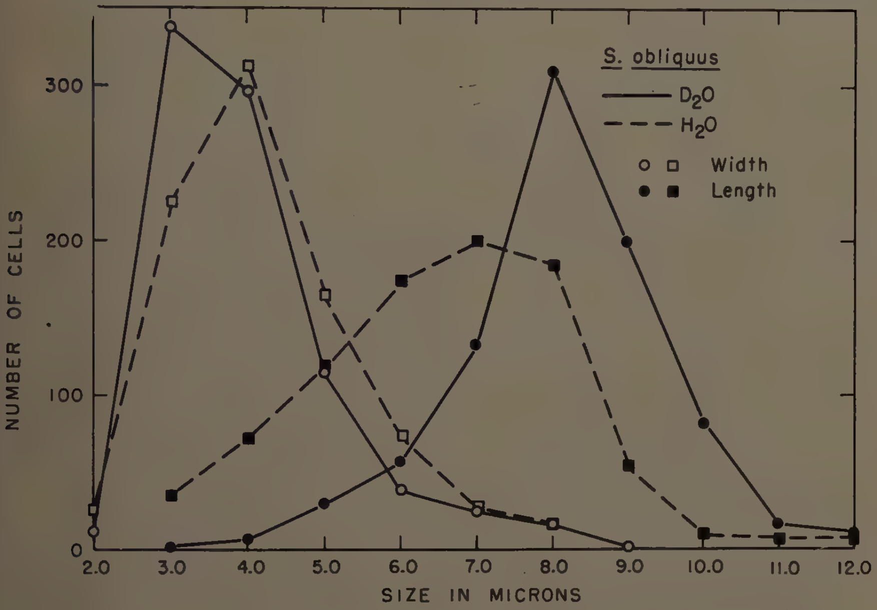

FIGURE 1. Size frequency distribution curves of hydrogen and deuterated *S. obliquus*.

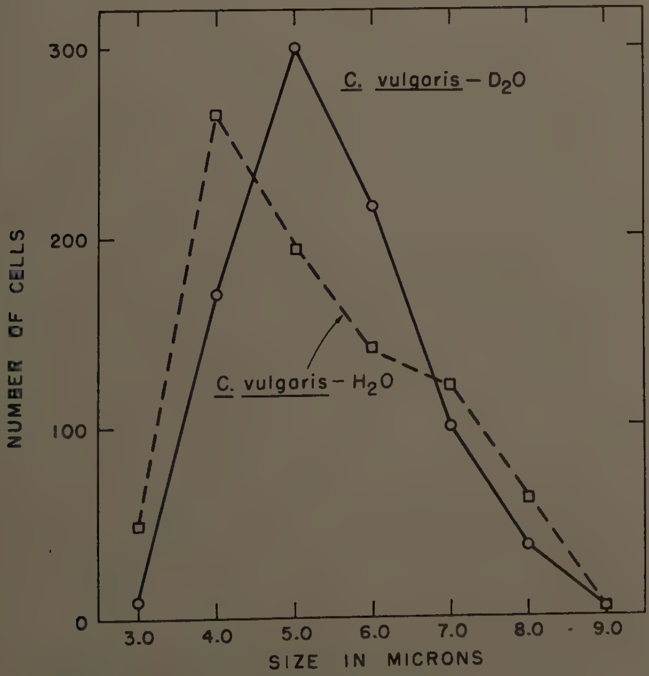

FIGURE 2. Size frequency distribution curves of hydrogen and deuterated *C. vulgaris*.

from both deuterated and ordinary cultures were multinuclear. However, a mitotic figure was seen with far greater frequency in deuterated cells than in hydrogen ones. This implies that a greater portion of the deuterated cells' life cycle is occupied with mitosis than is that of the hydrogen cells.

Another dissimilarity in internal structure between deuterated and nondeuterated cells is found in the chloroplasts. While both *S. obliquus* and *C. vulgaris* normally have single, simply-shaped chloroplasts, deuterated cells exhibit many variations in chloroplast shape and number.

Cytochemistry

The mode of fixation used prior to all cytochemical procedures was freeze-substitution. Cells were spread on a slide and quenched in liquid nitrogen. The slide with the cells frozen to it was then removed from the liquid nitrogen, laid flat, and flooded with absolute alcohol, or when lipid staining was anticipated, with 10 per cent formalin. Fixation periods of from 10 min. to 3 hours resulted in no perceptible differences in the appearance of cells, except that prolonged immersion in alcohol produced, especially in *C. vulgaris*, a progressive extraction of chlorophyll. Otherwise the fixed cells appeared lifelike.

In evaluating the results of staining and extraction procedures, the assumption has been made that fully deuterated compounds react to these tests in a manner resembling that of corresponding hydrogen compounds. More precise quantitative methods than those employed here would be required to determine the validity of this assumption.

Nucleic Acids

In order to determine the positions and to subjectively estimate the quantities of nucleic acids, the Feulgen[2] and the Azure B[4] methods were employed. Information obtained by these stains was combined with that from enzyme and from acid extractions. Early results of these and other nucleic acid tests will be reported elsewhere.[3]

We summarize here by saying that the areas of deuterated cells that react positively for nucleic acids are less localized than are those of the hydrogen-containing cells. This, as seen in FIGURE 3, is attributable not only to the greater nucleus size in deuterated algae but also to more widespread cytoplasmic staining in these cells. Furthermore, the intensity with which these nucleic acid-positive areas stain is greater in the deuterium-containing cells than in their hydrogen controls.

Proteins

Staining of proteins was effected by the Fast Green technique of Schrader and Leuchtenberger[5] at a *p*H of 1.2. These authors refer to Chapman *et al.*[6] as indicating that this technique should provide a simple, direct relationship between dye binding and free basic groups. However, various factors, as reviewed by Singer,[7] as well as the above mentioned isotope effect, may complicate the staining relationship. Nevertheless, the apparent nature of the staining differences reported below argues for their tentative acceptance.

The staining of unextracted cells was compared with that of cells that had

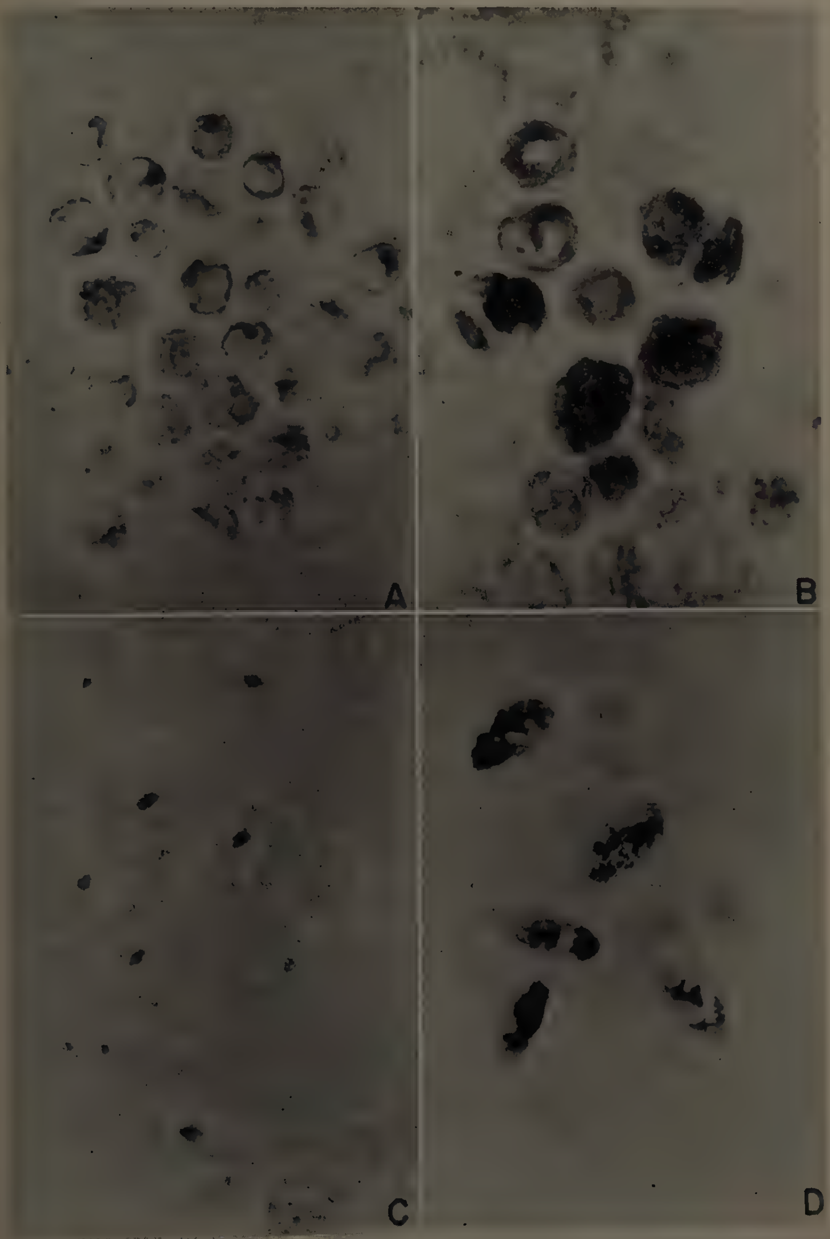

FIGURE 3. Cells stained for total nucleic acids by the Azure B technique: (A) ordinary *C. vulgaris*; (B) deuterated *C. vulgaris*; (C) ordinary *S. obliquus*; and (D) deuterated *S. obliquus*.

been previously extracted with pepsin in one-fiftieth normal hydrochloric acid, and this extraction procedure was controlled by treatment of slides with non-enzyme containing acid solutions.

As expected, this protein dye stained the cells far more uniformly than did the nucleic acid stains. The deuterated cells stained more intensely than corresponding hydrogen ones. As seen in FIGURE 4, the dye is far more concentrated in deuterated *C. vulgaris* than in ordinary hydrogen *C. vulgaris*. A comparison of sectioned cells shows that the apparently more intense staining of deuterated algae is not merely the result of a longer absorbing light path arising from the difference in cell size. A situation resembling that of *C. vulgaris* was found following Fast Green staining of *S. obliquus*. (FIGURE 4.) In most cells of deuterated *S. obliquus* the heavy cytoplasmic staining, which is concentrated chiefly in the chloroplast, obscures the nucleus. However, in some cells the nucleus may be seen (FIGURE 4). In nondeuterated *S. obliquus* there is less intense cytoplasmic staining and the nucleus is readily observed.

Lipids

In order to determine the distribution of nonmasked lipids, the Sudan black technique according to Leson[8] was employed. The specificity of results was confirmed by alcohol extraction either before or after staining. The general staining of deuterated cells of both *S. obliquus* and *C. vulgaris* indicated, as seen in FIGURE 5, some Sudanophilia throughout most of the cytoplasm. This background Sudanophilia was more intense in hydrogen than in deuterated cells. However, localized concentrations of Sudanophilia were more common in deuterated than in nondeuterated algae.

In *C. vulgaris* all deuterated cells reveal at least one highly Sudanophilic granule. Judging from their size and shape, these granules represent the nucleus. Some deuterated cells have one or more granules peripheral to the nucleus. These peripheral granules are often, but not always, associated with the chloroplast. Ordinary *C. vulgaris* shows few if any granules.

A similar situation obtains in Sudan stained *S. obliquus*. However, both deuterated and ordinary hydrogen cells show granules and practically all *S. obliquus* have a nucleus associated granule. The lowest level of Sudanophilia was observed in cells newly arisen from division. A far higher per cent of deuterated than of ordinary cells had this juvenile staining. As the cells became round preparatory to division, general cytoplasmic Sudanophilia rises, and the number of Sudanophilic droplets increases. This change is more pronounced in ordinary than in deuterated cells.

Carbohydrates

In order to compare the carbohydrate distribution of the algae, they were stained by the periodic acid-Schiff (PAS) method according to Hotchkiss.[9] Because it was anticipated that cell wall staining would interfere with examination of internal structures in intact algae, we deemed it necessary to section the algae. Consequently, algae after being quenched in liquid nitrogen were removed to absolute alcohol. The cells were centrifuged in the alcohol in order to form a pellet. The pellet was then xylene-cleared, recentrifuged, and paraf-

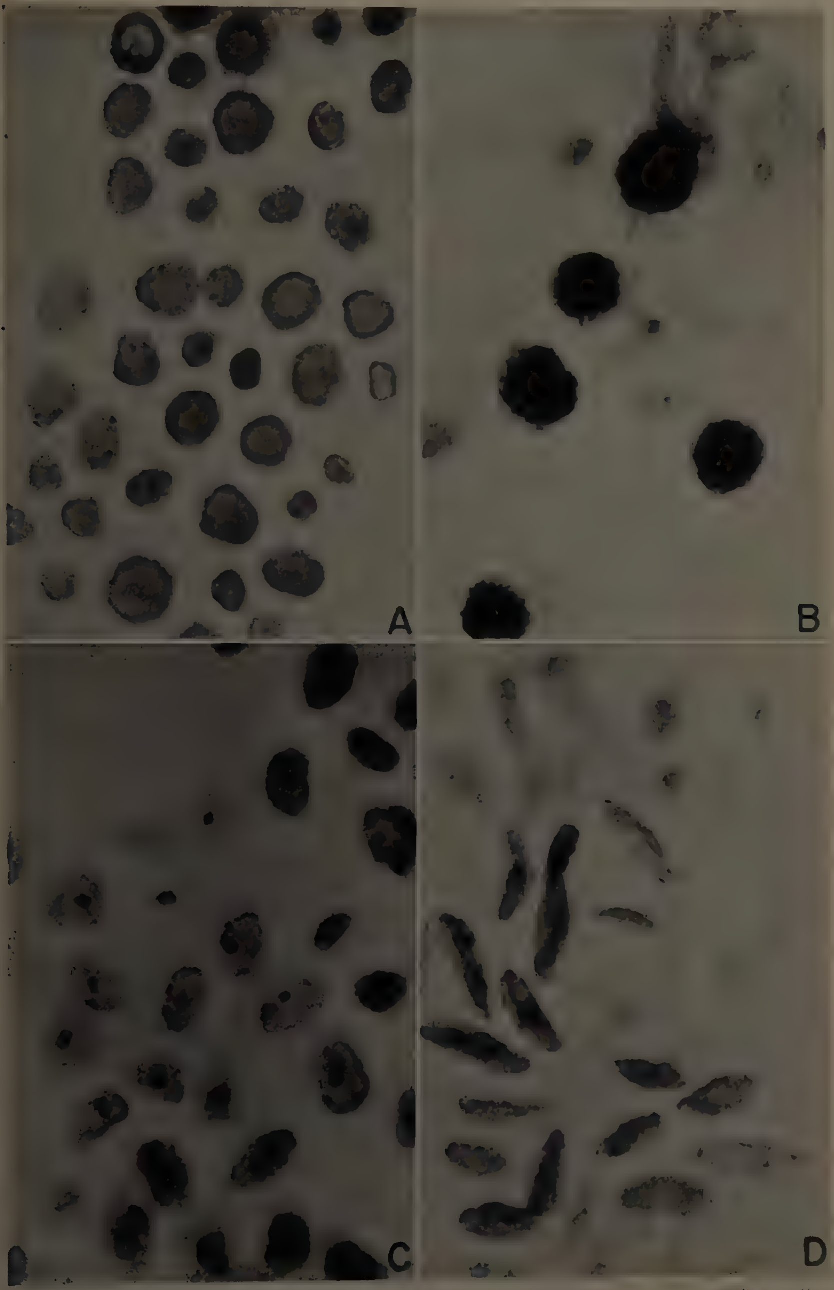

FIGURE 4. Algae stained for free basic groups by the Fast Green technique: (A) ordinary *C. vulgaris*; (B) deuterated *C. vulgaris*; (C) ordinary *S. obliquus*; and (D) deuterated *S. obliquus*.

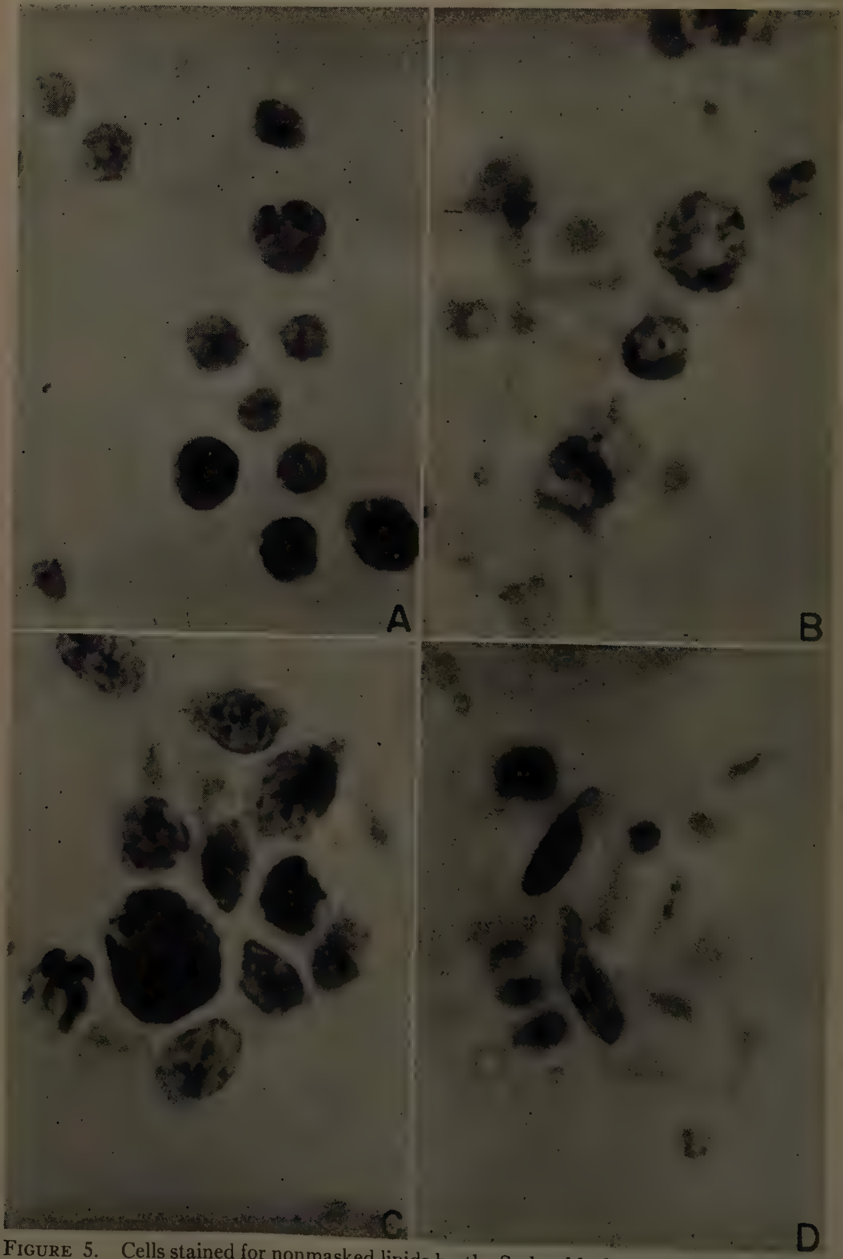

FIGURE 5. Cells stained for nonmasked lipids by the Sudan black technique: (A) ordinary *C. vulgaris*; (B) deuterated *C. vulgaris*; (C) ordinary *S. obliquus*; and (D) deuterated *S. obliquus*.

fin-imbedded in the usual manner. At present, only the study of *C. vulgaris* carbohydrates has reached a point where it can be reported.

The areas of heavy staining in *C. vulgaris* (FIGURE 6) are the cell wall and the chloroplasts. The edges of the chloroplasts stained especially heavily. Between these heavily stained areas were found other areas that stained weakly. The areas of deuterated *C. vulgaris*' walls that were PAS positive appeared to be thicker than corresponding areas in ordinary cells. Also the deeply staining areas of the chloroplasts were more intensely colored in the former cells than in the latter. Localized concentrations of carbohydrates that might be regarded as pyrenoid bodies were seen in the chloroplasts of deuterated cells with far greater frequency than in those of ordinary algae. However, a diffuse background of PAS-positive material was seen in most of the hydrogen *Chlorella*. This background staining was not seen with nearly as great frequency or intensity in the deuterated cells.

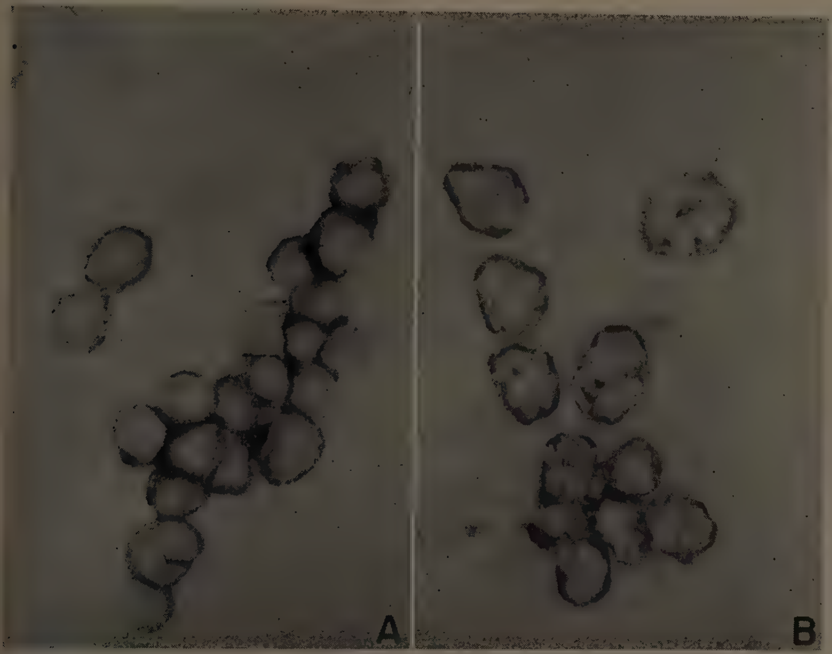

FIGURE 6. Cells stained for carbohydrates by the periodic acid-Schiff technique: (A) ordinary *C. vulgaris*; and (B) deuterated *C. vulgaris*.

When sections were digested with amylase at a *p*H of 6.7 prior to staining, the staining of all areas was reduced. This reduction in staining was uniform throughout the cell, but after two hours of such a digestion, no structure had lost all of its PAS-positive material. Staining material was also uniformly removed from sections that had been immersed in the *p*H 6.7 buffer without enzyme. However, three hours treatment with the buffer alone removed substantially less staining material from most areas than two hours treatment with the amylase.

Some PAS-staining structures appeared to be equally sensitive to treatment with buffer alone and with enzyme-containing buffer. Conspicuous among these were the pyrenoidlike concentrations. We regard buffer-extractable material as being composed of relatively low molecular weight saccharides. The additional material beyond these buffer-extractable substances that is removed by amylase treatment is regarded as starch. The PAS-positive substances that are not removed by prolonged exposure to amylase are thought to be relatively high molecular weight polysaccharides, mainly cellulose.

Discussion

These studies clearly indicate distinct differences in the morphology, and in the amounts and distribution of nucleic acids, proteins, lipids and carbohydrates in deuterated as compared to ordinary algae. In a general way the conclusions arrived at by the nucleic acid techniques and by the Fast Green protein method are parallel. There are relatively more acidophilic proteins and basiphilic nucleic acid present in the deuterated cells. Our findings in lipids and carbohydrates are more equivocal. These components are certainly distributed differently in deuterated algae, but the cytochemical methods employed here are incapable of determining whether the total quantities of lipid and carbohydrate are altered by deuteration.

The differences between deuterated and ordinary green algae might have resulted from either somatic adaptation or from genetic mutation. However, it is important to remember that the range of variation observed in the composition of *Chlorella* probably has not been exceeded with any other plant material.[10] Consequently, it is not possible to conclude that the changes in chemical composition observed in deuterated algae are the direct consequence of the replacement of hydrogen by deuterium. They may in fact result from the stress imposed by a generally unfavorable environment, and perhaps could be duplicated in ordinary water by unfavorable changes in the culture conditions. It also remains an open question whether the accumulation of relatively larger amounts of nucleic acid and protein in deuterated algae results from increased synthesis or reduced utilization, or is a reflection of a cell in distress.

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THE EFFECTS OF DEUTERIUM OXIDE ON CERTAIN MICROORGANISMS*

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Since the discovery of deuterium, several investigators have reported the inhibitory effects of the isotope in a variety of biological systems. This study interests itself in any phenotypic or genotypic changes deuterium might induce in the bacterial cell and attempts to correlate such changes with some particular action of the isotope.

One might imagine that phenotypic changes result from biochemical reactions immediately affected by deuterium. Moreover, the transient deuterium content of the cell might induce the occurrence of errors at the time the hereditary determinant replicates ultimately leading to permanent changes in the genotype of the cell. If the latter occurred, deuterium would be classified as a mutagenic agent.

Many theories have been advanced to explain the mechanism of mutagenesis. Although no one theory explains the action of all mutagenic agents, and while there is no proof that the mutational event is always the same phenomenon, the final effect must be the permanent alteration of the deoxyribonucleic acid (DNA), if one assumes that this molecule controls the flow of genetic information.

In some cases a temporary change in the DNA molecule will induce mutagenesis. Zamenhof and Griboff[1] found that, when special strains of *Escherichia coli* were grown in the presence of 5-bromouracil, there was considerable replacement of thymine by 5-bromouracil in the highly polymerized DNA of these cells. Such alteration of the DNA molecule induced not only phenotypic changes, but a high mutation rate and gene unstabilization.[2] Similar results were obtained by other investigators in studies with bacteriophage[3,4] and *Salmonella typhimurium*.[5]

Another change in the DNA molecule would be the replacement of hydrogen by deuterium. This would most likely occur during the synthesis of the molecule in the presence of deuterium *in vivo*. Such changes may upset the bonding and geometry of the DNA molecule so that at replication errors might occur.

In an early investigation of *Drosophila*, Zamenhof and Demerec[6] reported an inhibition of growth but no significant increase in mutation rate; similarly, the replacement of labile hydrogen atoms by deuterium upon dissolving the transforming principle in deuterium oxide did not change the transforming activity in *Hemophilus influenzae* (S. Zamenhof and G. Leidy, unpublished results). However, progress in the field of microbial genetics suggested the conduct of further study; indeed our preliminary work, reported last year, indicated that both phenotypic and genotypic changes could be obtained.[7]

PHENOTYPIC EFFECTS

Attention was first focussed on the phenotypic effects exhibited by several strains of *E. coli* and *Bacillus subtilis*, when grown in the presence of deuterium

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oxide (D_2O). The growth response of specific bacterial strains varied. In general, there was as much as a fivefold increase in the duration of the lag period and a threefold increase in the generation time; prolonged incubation did not result in a final cell number equal to that obtained in control cultures after 24 hours of incubation at 37° C. As one would expect, the inhibition decreased as the concentration of deuterium oxide in the media was reduced. No one strain grew well at concentrations above 80 per cent D_2O and most were not inhibited by concentrations below 50 per cent D_2O. No extensive death was noted upon exposure to deuterium oxide.

Growth of bacteria in the presence of deuterium was enhanced by agitation, more complex media, or by the incorporation of 0.5 per cent sodium chloride in the medium. Perhaps the sodium chloride has an effect on the permeability that helps the cell overcome some but not all of the inhibitory effects of deuterium.

Cells grown for long periods of time in D_2O media gave no indication of adaptation to better growth in its presence.

DEUTERIUM-RESISTANT MUTANT

From strain I (*E. coli* 15, thymine$^-$ mutant), an apparent spontaneous mutant, I/D, more resistant to deuterium oxide, was obtained. The mutant could be maintained after several transfers on nutrient agar free of D_2O; therefore, it represented not a transitory adaptation but a true mutation. The frequency of this spontaneous mutant was of the order of 10^{-4} and could be increased 300 times by ultraviolet irradiation. It was easier to demonstrate its reduced sensitivity to deuterium on nutrient agar than in nutrient medium. Attempts to obtain such a mutant from other strains of *E. coli* and *B. subtilis* have been unsuccessful.

GENOTYPIC EFFECTS

Forward Mutations

The cultivation of some bacterial strains in the presence of high concentrations of D_2O led not only to inhibition of growth but to the induction of mutants of different types.

Pinpoint Colonies. Mutants that produced small colonies on a synthetic agar and on nutrient agar and required further enrichment for full colony formation reverted to the parent type at a high rate.

Stable Auxotrophs. These mutants formed normal-sized colonies on nutrient agar but did not grow on a synthetic agar.

Unstable auxotrophs A. These mutants form normal-sized colonies on nutrient agar but do not grow on synthetic agar. They revert at a high rate to the parent type which is able to grow on synthetic agar.

Unstable auxotrophs B. These mutants form small-sized colonies on nutrient agar and are unable to grow on synthetic agar. They revert at a high rate to the parent type, which is able to form full-sized colonies on nutrient and synthetic agar.

Backward Mutations

Preliminary experiments have been made to investigate further the mutagenicity of deuterium by examining the backward mutation of strains at particular assayable loci. Five strains were examined, two of which showed an increase in the mutation frequency over the spontaneous level. In Sd/4 (*E. coli*, streptomycin-dependent mutant) the streptomycin-sensitive mutant independent of streptomycin appeared 25 times more frequently in D_2O than spontaneously. The mutation of strain I to thymine independence was increased up to 100-fold.

These studies seem to indicate that the mutagenic action of deuterium may be specific in that it affects some loci and not others. However, much more work must be done with other strains and other loci before the extent of the mutagenicity of deuterium can be fully evaluated. Specific loci may be more sensitive to hydrogen-deuterium exchanges.

CHEMICAL ANALYSIS

In order to get some information concerning the incorporation of deuterium into DNA, an analysis was made of the amount of deuterium incorporated into one of the bases, namely thymine. Of the possible four nonlabile hydrogens that could be replaced by deuterium, two actually were. There is an indication that the replacement of hydrogen may all be in the methyl group.[8]

Strain I was able to utilize thymine labeled in such a way as well as normal thymine. No detectable phenotypic or genotypic effects were induced by deuterium containing thymine. The genotypic effects previously discussed are most probably due to the more extensive replacement of hydrogen by deuterium in the DNA molecule, or possibly to the effect deuterium induces on enzymes involved in DNA synthesis.

DEOXYRIBONUCLEIC ACID CONTAINING DEUTERIUM

The exposure of such DNA to ultraviolet irradiation and other chemical and physical agents would indicate whether deuterium incorporation affected the stability of the molecule. In addition, the biological activity of such DNA could be determined by transformation studies.

The effect of low doses of ultraviolet irradiation on the survival of cells grown in the presence of deuterium was studied first. Greer and Zamenhof[9] found that cells containing 5-bromouracil were 20,000 times more sensitive to ultraviolet light than were control cells. The results of a preliminary experiment with D_2O did not show such spectacular differences. An interesting result was that the most sensitive cells were those that had been grown in D_2O medium and subsequently washed and irradiated in H_2O saline; they were 50 times more sensitive than control cells. Work on this subject is being continued, in particular, on the sensitivity of DNA containing both hydrogen and deuterium.

SUMMARY

The growth of several strains of *E. coli* and *B. subtilis* was inhibited by the presence of D_2O; the degree of inhibition exhibited by each strain was specific.

The addition of 0.5 per cent NaCl to the D_2O media decreased the inhibition of growth. A deuterium-resistant mutant was obtained from one strain of *E. coli.* The incorporation of deuterium induces not only phenotypic but also genotypic changes in microorganisms. The effects induced by deuterium depend, however, on the genotype of the strain. The isotope appears to be mutagenic for some strains and some loci but not for others. Various types of forward mutations were obtained in some of the bacterial strains tested and the frequency of backward mutation was increased in two strains exposed to deuterium. Thymine containing deuterium, possibly in its methyl group, is not capable of inducing any detectable changes in a thymine requiring mutant. Cells, grown in D_2O media and subsequently washed and irradiated in H_2O-saline, are more sensitive to ultraviolet irradiation than control cells.

ACKNOWLEDGMENTS

I am grateful to S. Zamenhof for suggesting the problem to me and for many helpful discussions. I also thank Laura Ponticorvo of D. Rittenberg's laboratory for the deuterium analysis of thymine.

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CULTIVATION OF MICROORGANISMS IN HEAVY WATER*

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In recent years a number of reports have appeared in the literature to the effect that heavy water in high concentrations almost completely inhibits the autotrophic growth of algae.[1-4] These reports are in agreement in finding "that heavy water inhibits growth and, in concentrations approaching 100 per cent, no growth occurs."[4] The early results of Reitz and Bonhoeffer[5] along similar lines thus appeared amply confirmed. The upper limit for the successful growth of algae was apparently firmly established in the range of 60 to 75 per cent D_2O. Within the past year, however, Chorney and his co-workers[6] have described the successful culturing of a number of species of green algae in 99.6 per cent D_2O. Although growth rates were reduced, they were nevertheless high enough to encourage further physiological studies and exploration into the large scale culture of *Scenedesmus obliquus*.

The work reported here supplements and extends the original successful work with the green algae. A preliminary account of these experiments has appeared elsewhere.[7] The growth rates previously reported[6] have been greatly improved by appropriate changes in the composition of the nutrient media. Two other species of green algae and three species of blue-green algae have also been cultured at D_2O concentrations in excess of 99 per cent. In the course of our survey work we have also encountered a few species of algae whose distaste for D_2O has so far prevented growth in media containing more than 75 per cent deuterium. These experiments will also be described. On the other hand, preliminary data on the mass culture of *S. obliquus* indicate that high yields of fully deuterated organisms can be obtained on a continuous basis.

Fully deuterated organisms and the fully deuterated compounds formed by them by biosynthesis suggest a variety of experiments of a fundamental nature. It now becomes practicable to investigate the deuterium kinetic isotope effect in living organisms. Since the rate of various reactions will be affected by isotope substitution to different degrees, a unique situation presents itself for the study of the kinetics of reactions in living organisms and for locating rate-controlling steps in biological systems. Utilization of fully deuterated substrates such as sugars and amino acids may contribute to an understanding of enzyme-substrate interactions, as in the work of Abeles and his co-workers,[8] and fully deuterated enzymes may likewise have special value in the study of enzyme function. Fully deuterated proteins and nucleic acids should be useful in the study of coil-helix transformations, for the study of the role of the hydrogen bond in maintaining the structure of these substances, and in determining the effect of lipid-lipid side-chain interactions on the stability of proteins. Isotopically altered compounds and organisms may provide an indespensable key to the successful application of electron spin resonance and nuclear

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* Based on work performed under the auspices of the United States Atomic Energy Commission, Washington, D.C.

magnetic resonance techniques to biological systems. The resolving power of density gradient ultracentrifugation should be considerably increased with macromolecules substituted with D, C^{13}, N^{15}, and O^{18}; genetic transfer in bacteria can perhaps be studied in this way. *Chlorella vulgaris* grows well in D_2O with N^{15} in the nitrogen source, and it is altogether likely that algae will be able to biosynthesize compounds fully substituted with any combination of D, C^{13}, N^{15}, and O^{18}. The research possibilities opened up by isotopically substituted organisms and compounds, particularly with deuterium, are clearly numerous and varied.

With the fully deuterated compounds available from the algae grown in D_2O, it becomes quite feasible to grow more demanding organisms in an essentially fully deuterated environment, and experiments along these lines are also described here.

TABLE 1
THE GROWTH OF ALGAE IN D_2O: A SUMMARY OF RESULTS

Species	Source	75%	90%	99.6%	Comments
Chlorella ellipsoidea	Tamiya	+	+	+	
C. pyrenoidosa	Emerson	+	+	+	
C. pyrenoidosa	Sorokin	+	−		High temperature strain
C. vulgaris	Emerson	+	+	+	
Scenedesmus obliquus	Gaffron	+	+	+	
Anacystis nidulans	Gaffron	+	−		At 37° C.
Fremyella diplosiphon	Starr-481*	+	+		In progress
Nostoc commune	Starr-584*	+	+	+	
Phormidium luridum	Starr-426*	+	+		In progress
P. sp.	Starr-485*	+	+	+	
Plectonema calothricoides	Starr-598*	+	+	+	
Navicula pelliculosa	Starr-645*	+	−		
Phaeodactylum tricornutum (*Nitzschia closterium* f. *minutissima*)	Starr-646*	+	−		
Porphyridium cruentum	Starr-161*	+			In progress

* Obtained from the Culture Collection of Algae at Indiana University. The number is the catalog number.

Experimental Methods

TABLE 1 lists the species of algae used in these experiments. Cultures obtained from the Culture Collection of Algae at Indiana University, Indianapolis, Ind., are starred with the catalog number. The conditions for growth of the algae were similar to those previously reported;[6,7] most experiments reported here were conducted in 125 ml. flasks containing about 60 ml. of nutrient medium. Aseptic techniques have maintained cultures in uni-algal condition, but there has been bacterial contamination, probably because of the ground-glass joint on the culture flasks. Deuterated green algae have been preserved for periods up to 12 months at 4° C. on deuterated agar slants* containing deuterated glucose. Glucose is essential since the organisms fare poorly when grown autotrophically on deuterated agar slants. Experiments on the preservation of adapted blue-green algae on deuterated slants are in progress. These techniques have made it possible to maintain deuterated cultures by more or less conventional procedures.

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* The agar was prepared by repeated solution and recovery of agar in 99.6 per cent D_2O. Exchangeable hydrogen was replaced with deuterium.

Growth has been followed by measurements of dry weight, packed cell volumes, and cell counts of the algae. The nutrient media used are listed in TABLE 2.

TABLE 2
NUTRIENT MEDIA FOR THE GROWTH OF ALGAE

Component	*Chlorella* media			*Scenedesmus* medium	Blue-green algal medium "Ac"[19]
	1*	2*	3†		
		Grams per liter			
KNO_3	1.25	1.25	1.25		2.0
$NaH_2PO_4 \cdot H_2O$	1.00	1.00		0.20	
KH_2PO_4			1.25		
K_2HPO_4	0.25	0.25			0.27
$MgSO_4$	0.125	0.125	0.50	0.48	0.125
ND_4NO_3				1.00	
$KHCO_3$				0.20	
$Ca(NO_3)_2 \cdot H_2O$				0.03	
NaCl		2.0		0.02	0.23
$CaCl_2$			0.08		0.055
$FeSO_4 \cdot 7H_2O$	0.006	0.012	0.02	0.03	0.005
NaOD			to *p*H 6.4		
DCl					to *p*H 6.6
		Parts per million			
B	0.5	0.5	0.5	1.0	0.5
Mn	0.25	0.25	0.25	0.50	0.25
Zn	0.05	0.05	0.05	0.10	0.05
Cu	0.02	0.02	0.02	0.04	0.02
Mo	0.05	0.05	0.05	0.10	0.05
Co			0.01		0.01

* For *C. vulgaris*.
† For *C. ellipsoidea*, from Sorokin and Myers.[18]

Adaptation Phenomena

Once algae are adapted to growth in D_2O they grow well and at a good rate. However, the adaptation process itself presents many puzzling features and the factors leading to successful growth in D_2O are still for the most part obscure. We describe here some experiments of a more or less miscellaneous nature that may clarify some aspects of the situation.

Chorney *et al.*[6] noted the lengthy adaptation period required before algae grow in D_2O. As the concentration of D_2O in the medium is increased above 75 per cent, the lag period is progressively increased and the maximum density attained by the culture decreased. FIGURE 1 illustrates the results of a serial subculture of *Chlorella ellipsoidea* (Tamiya) in increasing concentrations of

D_2O. The linear growth rate was progressively lowered and the level of maximum cell density of the culture was precipitously depressed with increasing D_2O concentration. In the cultures at 90 and 99 per cent D_2O there was an initial lowering of *p*H of about 0.1 unit before a gradual climb to the region of neutrality was observed. A logarithmic plot of growth at 99 per cent D_2O showed a lag of about 4 days before the logarithmic phase began.

During adaptation of a considerable variety of microorganisms we have noted that the *p*H frequently falls to very low levels. FIGURE 2 illustrates the results of some experiments in which adaptation of *S. obliquus* was studied in nutrient media containing different amounts of phosphate. In high phosphate concentrations, as the number of cells increases, the *p*H rapidly drops, indicating that acid production may be associated with cell division. As the *p*H approaches 4, the cultures become white and turbid because of the presence of very large, almost colorless cells. These cultures contain much larger concentrations of glycollic acid than are found in fully adapted cultures in D_2O. The glycollic acid concentration in acidic cultures that fail to adapt may approach 50 mg./l., but whether it is excreted or arises from cell lysis remains to be determined. Experiments have been carried out in which the *p*H of the adapting cultures was controlled by periodic addition of alkali. Although these cultures fared better in the beginning, their ultimate fate was not significantly different.

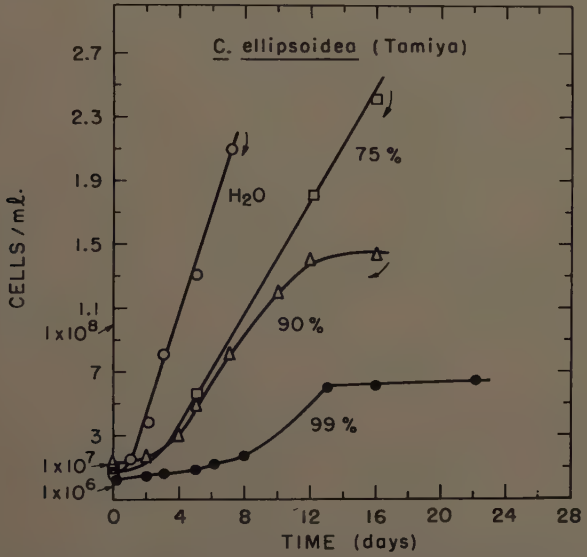

FIGURE 1. The growth of *Chlorella ellipsoidea* in increasing concentrations of D_2O. Cells per milliliter are plotted on a linear scale against time. The arrows indicate the point at which the culture was used to inoculate the succeeding culture. Note the marked reduction in the level of maximum cell density, indicating, perhaps, changed nutritional requirements.

The effects of changes in the nutrient media on the adaptation of *S. obliquus* to growth in D_2O have been studied in some detail. Cultures of this organism obtained from H. Gaffron, of the University of Chicago, Chicago, Ill., adapt much more readily than the same organism obtained from the Indiana University collection. Adaptation in the regular nutrient medium, which is quite suitable for continued growth in D_2O, was erratic. Replacement of the usual 5 per cent CO_2-95 per cent N_2 with 5 per cent CO_2-95 per cent air slowed the rate of *p*H drop and cell enlargement, but the end result, white, nongrowing cultures, remained unchanged. A similar result was obtained when a nutrient solution with half the usual concentration of salts was used. When the standard medium was used but with 0.10 gm./l. of ND_4NO_3 as the nitrogen source,

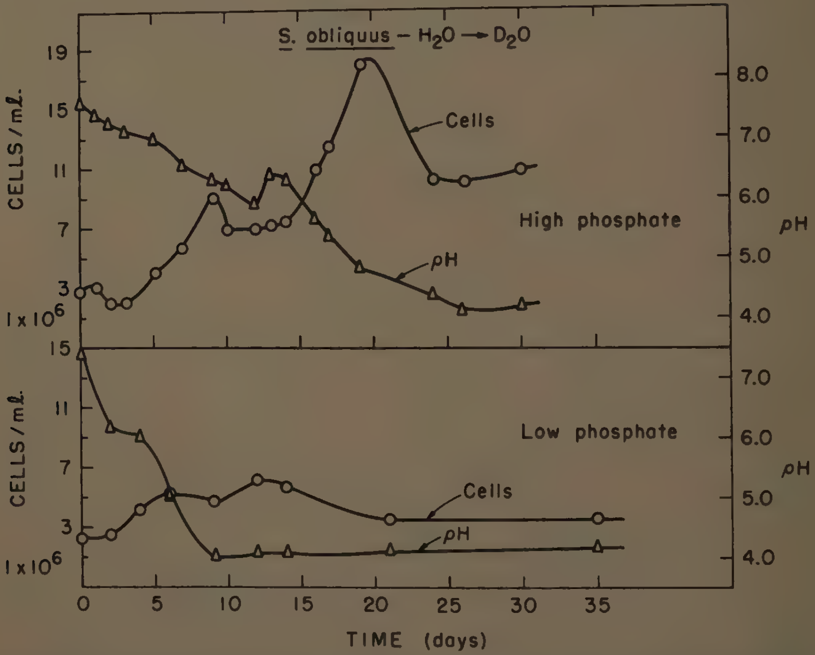

FIGURE 2. Graphs illustrating the acid production of *Scenedesmus obliquus* upon direct transfer from an H_2O nutrient medium to D_2O nutrient media. The "low phosphate" medium was that of TABLE 2 and the "high phosphate" medium was the same with an added 1.0 gm./l. of K_2HPO_4.

the characteristic *p*H drop did not occur, and the *p*H rose to 7.0 after 16 days. However, no adaptation occurred, more extremely large cells than normal were present, and the green color also eventually faded. The addition of ATP to the regular medium was without apparent effect on the adaptation.

A characteristic feature of adapting cultures is the presence of greatly enlarged "monster" cells. We have examined the cytochemistry of these cells in some detail. They contain extraordinarily large amounts of nucleic acids and show other unusual features that will be described elsewhere. (E. Flaumenhaft, S. M. Conrad, and J. J. Katz, unpublished data). We are under the impression that adapted organisms do not arise from these monster cells, but that the enlarged cells result from failure to adapt.

Unlike *S. obliquus*, *C. vulgaris* and *C. ellipsoidea* do not exhibit extreme sensitivity to direct transfer to D_2O from H_2O cultures. We have performed this experiment on numerous occasions with these two species and have always obtained growth after inhibition times of the order of four days (with *Chlorella* media 1 and 3; 2 is not conducive to adaptation). If *Scenedesmus* or *Chlorella* are transferred from an H_2O culture directly to a 99.6 per cent D_2O nutrient medium, the cells continue to grow to "division" size but division is strongly inhibited. It appears as if the growth and division mechanisms have become dissociated in some degree.[9] After a few days *Chlorella* divides, but *Scenedesmus* has considerably more difficulty and extremely large cells result. Nevertheless in fully adapted *Scenedesmus* the cells are somewhat smaller than normal. Once the block to cell division has been overcome its effect is no longer apparent.

Although serial subculture is not required for the adaptation of *C. vulgaris* or *C. ellipsoidea* to growth in D_2O, it does play a useful role in the adaptation of refractory or fastidious algae. All the blue-green algae that we have induced to grow in D_2O have been adapted by serial subculture, and we have never succeeded in growing a blue-green alga in D_2O by direct transfer from H_2O. All of the algae we have worked with grow at 75 per cent D_2O; if they can be persuaded to grow at 90 per cent, then there is a high probability that growth in 99 per cent D_2O will occur with no serious difficulties.

We can offer no facile explanation for success in culturing algae in D_2O where others have reported failure. The nutritional requirements of algae even in H_2O are far from a closed subject. At the present time the culture of algae in high concentrations of D_2O remains more of an art than a science.

Laboratory Culture of Algae

Growth rates. FIGURE 3 shows typical growth curves in 99.6 per cent D_2O for *S. obliquus* and *C. vulgaris*; for *S. obliquus* the salt medium listed in TABLE 2 was used, and for *C. vulgaris* the *Chlorella* medium 1, also listed in TABLE 2. Compared to previously published growth rates, the linear growth rates of these organisms in vessels of similar geometry have been increased by a factor of three for *S. obliquus* and two for *C. vulgaris*. An additional increase in growth rate of about 25 per cent was obtained for *C. vulgaris* by using *Chlorella* medium 2 (TABLE 2). The maximum culture densities obtained with these nutrient media are in the vicinity of 8 to 10 gm./l. (dry wt.) for *Scenedesmus* and about 4 gm./l (dry wt.) for *C. vulgaris*. The growth rate of *C. vulgaris* was not greatly changed by addition of 10^{-4} M Li^+, total substitution of N^{14} by N^{15}, or by replacement of 5 per cent CO_2-95 per cent N_2 by 5 per cent CO_2-95 per cent He. As might be expected, there was very little growth in a potassium-free nutrient medium.

We have not made a systematic study of the effect of changes in individual salt concentrations on the growth of *Scenedesmus*. A marked improvement in growth was noted when additional ammonium nitrate was added to the previously reported nutrient medium,[6] but the best growth and appearance (a greenness approaching that of H_2O cultures) were obtained with the added ND_4NO_3 and doubled concentrations of the other salts. Recently the concentration of calcium has been reduced, without depression of the growth rate, to minimize the formation of a precipitate in the nutrient solution.

FIGURE 4 shows logarithmic plots of cell count versus time as observed in *S. obliquus*, *C. vulgaris*, and the blue-green alga *Plectonema calothricoides*. Individual filaments were counted in, following the growth of *Plectonema*; the filaments averaged only about 6 to 10 segments and the average length did not appear to change during the course of the experiment. TABLE 3 summarizes k* values and doubling times. Doubling times for the green algae are generally about 35 to 40 per cent of the values ordinarily observed for the same algae in H_2O.

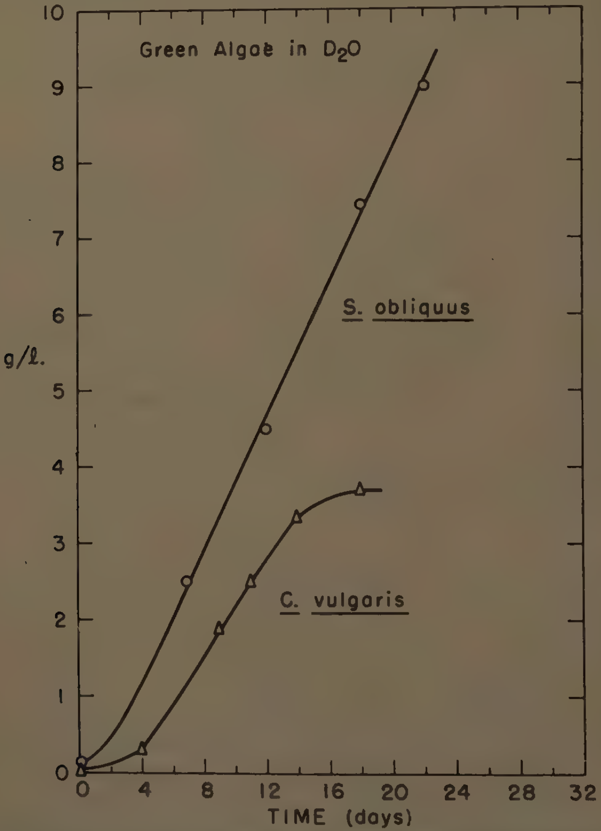

FIGURE 3. Growth curves for *Scenedesmus obliquus* and *Chlorella vulgaris* in nutrient media containing 99.6 per cent D_2O. Packed cell volumes were converted to gm./l. (dry weight) for this plot. Culture conditions were analogous to those given in Chorney *et al.*[6]

The growth of *Plectonema* (FIGURE 4) appears especially tortured in D_2O and a doubling time of 5.9 days was observed. The combination of 99.6 per cent D_2O and continuous exposure to light eventually causes a brown coagulum to form and the culture often dies. It appears necessary to use heavy inoculations in order to shade the algae if *Plectonema* is to be grown under conditions

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* The symbol k is defined by the expression $(dN/dt) = kN$, where N is the number of cells and t is the time. In this paper, k is given in units of ln per day.

of continuous illumination. Growth is considerably improved at 31 to 32° C., but as yet we have no quantitative data on the growth rate in this temperature region. FIGURE 5 compares by phase contrast photomicrography specimens of *P. calothricoides* grown in H_2O and in 99.6 per cent D_2O. In D_2O the filaments of algae are irregularly contorted and are rigid with short, knobby segments. The structure in H_2O is much more graceful and the segments are smooth cylinders.

Qualitative studies on various species of algae. As indicated in TABLE 1, a number of species of algae in addition to those already discussed have been

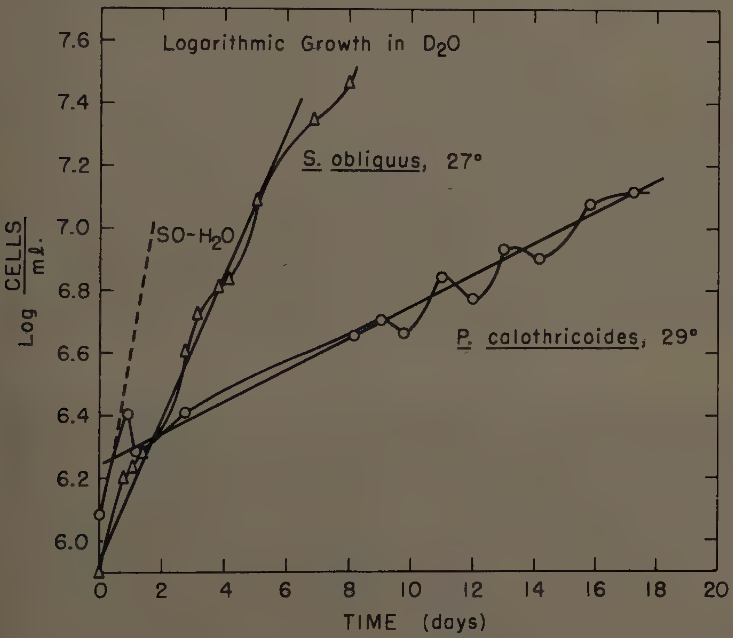

FIGURE 4. Logarithmic plot of cells per milliliter versus time for *Scenedesmus obliquus* and (filaments per milliliter) for *Plectonema calothricoides*. These growth rate data are obtained from dilute suspensions where light is not rate-limiting. The dotted line represents the rate for *Scenedesmus obliquus* in H_2O for comparison.

TABLE 3

GROWTH RATES OF VARIOUS ALGAE IN H_2O AND D_2O AT 27° C.

Species	Medium	Doubling time (days)	*k* (ln/day)
S. obliquus	H_2O*	0.53	1.3
S. obliquus	D_2O*	2.5	0.27
S. obliquus	D_2O†	1.3	0.53
C. vulgaris	D_2O‡	1.2	0.60
P. calothricoides	D_2O§	5.9	0.12

* Nutrient medium of Chorney *et al.*[6]

† *Scenedesmus* medium of TABLE 2.

‡ *Chlorella* medium 2 of TABLE 2.

§ Nutrient medium "Ac" of TABLE 2.

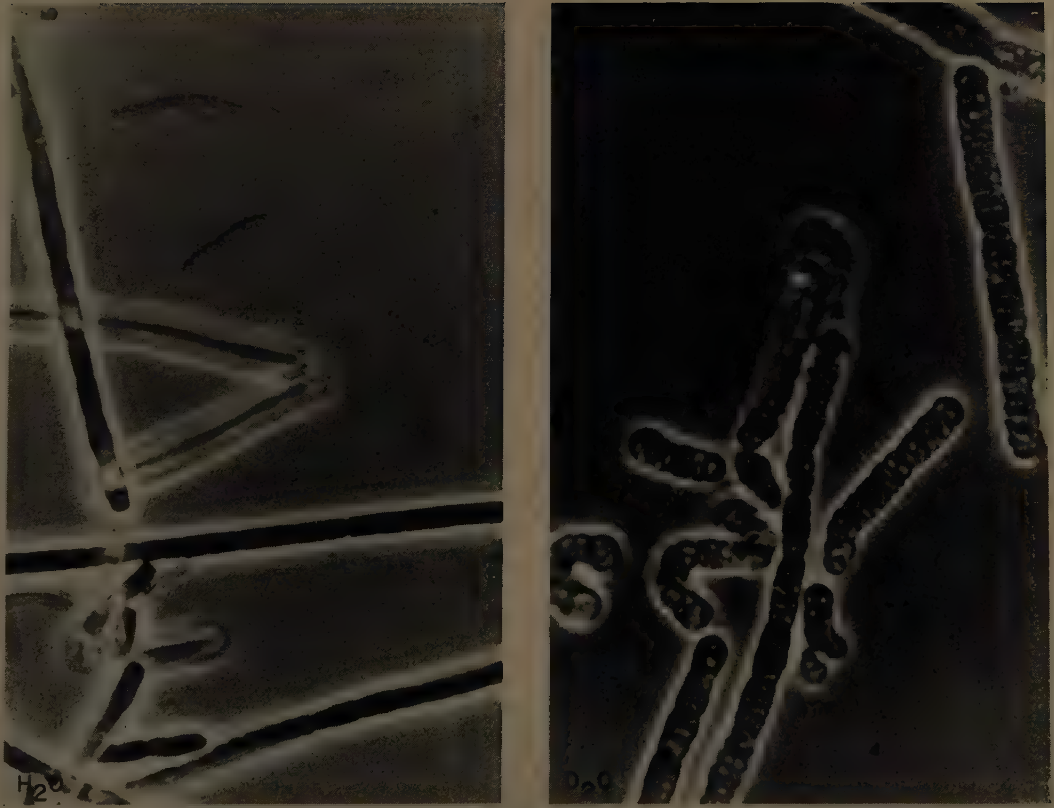

FIGURE 5. Phase contrast photomicrographs of the blue-green alga *Plectonema calothricoides* grown in H_2O (*left*) and 99.6 per cent D_2O. The filaments in D_2O (*right*) are irregularly contorted, appear rigid, and are generally much shorter than in H_2O.

examined for adaptability to growth in D_2O. These studies have met with varied success, and the results of these qualitative studies will be discussed with respect to each individual species. In these experiments decisions as to whether growth had occurred were made on the basis of visual and microscopic examination.

Phormidium sp. This species grew well in "Ac" nutrient medium in H_2O and was serially subcultured into 75, 90, and 99.6 per cent D_2O at 27 to 29° C. The H_2O culture was inoculated from an agar slant and subsequent inoculations were heavy and made from very opaque liquid cultures. The course of adaptation was uneventful. FIGURE 6 is a phase contrast photomicrograph of this species in H_2O and in about 99.2 per cent D_2O. In D_2O the filaments appear rigid and the segments are markedly misshaped. After showing good growth in 99.2 per cent D_2O, further subculture in D_2O produced no growth and the culture was lost.

Phormidium luridum. This species grew very well in the H_2O "Ac" nutrient medium when inoculated from an agar slant, and on the tenth day a portion of the H_2O culture was used to inoculate a 75 per cent D_2O medium. On the fourth day this culture was already showing good growth, and on the tenth day a portion of the 75 per cent D_2O culture was used to inoculate a 90 per cent D_2O medium. This 90 per cent D_2O culture showed essentially no growth for 32 days and was then transferred from the mechanical shaker at 27° C. to a shaker maintained at 32° C. On the second day, at 32° C., growth began. The results of this experiment were confirmed by setting up two cultures in 90 per cent D_2O from the 75 per cent D_2O culture. The one kept at 27° C. did not show any growth, while the one maintained at 32° C. showed growth almost immediately. This sensitivity to temperature may be the reason *Phormidium* sp. ceased to grow in 99 per cent D_2O as described above. Apparently there is a critical temperature for these cultures in D_2O below which they will not show growth.

Nostoc commune. This colonial blue-green alga was also serially subcultured in "Ac" nutrient medium from H_2O in 99.3 per cent D_2O. The entire series of cultures took about three months, since this species grew very slowly. It was estimated that the doubling time in 99 per cent D_2O for this organism is of the order of 3 weeks. During growth in both H_2O and D_2O the media remained perfectly clear. The clumps of algae formed in D_2O were larger than those in H_2O and somewhat less blue in color.

Fremyella diplosiphon. After transfer from an Indiana Culture Collection agar slant to H_2O "Ac" nutrient medium, this species showed little growth activity for 4 or 5 days, and then grew well to give a purplish-brown culture. A portion of this culture was used to inoculate a 75 per cent D_2O "Ac" nutrient medium. After a few days, the inoculum turned a yellowish-green in color, and remained in this condition for about two weeks without any growth. After a further two-week period the green color of the culture deepened as growth began, and after 3½ weeks this 75 per cent D_2O culture was a deep blue-green color and apparently growing well. A subculture into 90 per cent D_2O produced no growth after one month. Two other subcultures into 90 per cent D_2O were made from a second 75 per cent D_2O culture. One shaken at

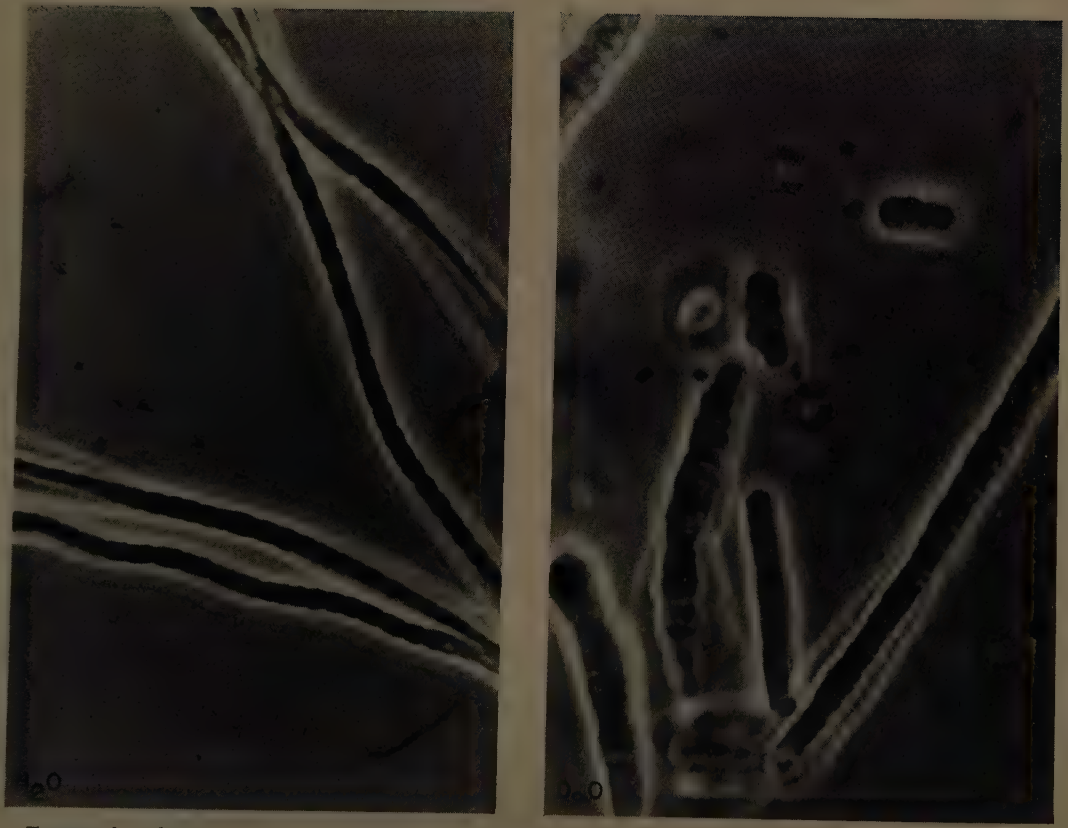

FIGURE 6. Phase contrast photomicrographs of the blue-green alga *Phormidium* sp. grown in H_2O (*left*) and 99.2 per cent D_2O. In D_2O (*right*) the segments are wider and variable in size and appear striated. The filaments are generally much shorter in D_2O than H_2O.

27° C. showed growth after two weeks while the other at 32° C. showed no growth after a month. The cultures of these organisms in D_2O are more typically blue-green in color than the original brownish culture in H_2O. Reinoculation of the D_2O cultures into H_2O produce a culture typical of the original organism.

Anacystis nidulans. This species, a thermophilic blue-green alga, was successfully cultured at 39° C. in a 75 per cent D_2O nutrient medium consisting of a mixture of D_2O "Ac" medium and the H_2O "C" medium of Kratz and Myers.[10] Attempts to culture this organism at higher D_2O levels were unsuccessful. A similar unsuccessful result was obtained with a high temperature strain of *Chlorella pyrenoidosa* (Sorokin) that was cultured in *Chlorella* medium 3. Our experiments with these two high temperature strains of algae were designed to eliminate the hydrogen-containing chelating agents as adaptation to D_2O progressed. It may be however, that the combined upsets in metabolism caused by removal of chelating agents and increasing D_2O concentration led to the negative result. We believe that better results with these high temperature strains will be obtained when completely deuterated chelating agents become available. Meanwhile, exploratory experiments with ordinary chelating agents are in progress.

Diatoms. Both *Phaeodactylum tricornutum* (*Nitzschia closterium* f. *minutissima*) and *Navicula pelliculosa* grow readily in 75 per cent deuterated artificial sea water media[11-13] but cultures could not be sustained at more elevated D_2O levels. A possible factor in the inability to adapt to higher concentrations of D_2O is indicated by the tendency of these diatoms to clump in deuterated media, even at low cell densities. These growth experiments were conducted at 15° C. without addition of soil extracts or other organic substances. Efforts to adapt these diatoms to higher deuterium levels are continuing.

Porphyridium cruentum. This species, a red alga, has been grown in an inorganic salt medium (J. M. Twarog and E. I. Rabinowitch, personal communication) in 75 per cent D_2O. Initial attempts to obtain growth at higher D_2O concentrations have failed, but a determined effort has not yet been made.

Large-scale culture of S. obliquus in D_2O. To enable us to isolate larger amounts of deuterated substances such as sugars, amino acids, chloroplast pigments, nucleic acids, and proteins, we have successfully mass-cultured *S. obliquus* in 99.6 per cent D_2O. FIGURE 7 shows the type of apparatus that has proved most useful. Five liters of algal culture are contained in a rectangular Lucite box held horizontally in a rocking metal frame. Light of an intensity of about 1000 ft.-ca. is supplied from below and the culture is maintained at a constant temperature by circulating cooling water through channels cut in the base of the box. At 25° C., the growth rate in terms of packed cell volume is about 1.3 ml./l./day, which corresponds to 0.33 gm./l./day (dry wt.). The unit illustrated in FIGURE 7 has operated continuously at this production rate for over three months. Harvests of 1-l. size are made every fourth day.

The results obtained in the rocking box are considerably improved over those reported earlier[7] for growth in an 8-l. upright Lucite box in which agitation was obtained by pumping the culture medium. The geometry of the 8-l. upright system was similar to the rocking system, but the culture was maintained vertically rather than horizontally; that is, the dimensions of the surface area were 1 inch × 28 inches and the culture depth was 17 inches. Lighting was from the side. Pumping the culture does not give fully adequate agitation; foam forms at the surface from the CO_2-N_2 gas mixture bubbled through the culture; and, more important, the growth rate fell with time so that after several months it had decreased to less than one fifth the initial rate. The bacteria level also became quite high in these "vertical" boxes. Consequently, such units and similar 4-l. doughnut-shaped units[14] have been largely abandoned in favor of the "horizontal" rocking boxes. Full details of the operation of the rocking boxes and the chemical fractionation of the deuterated algae will appear in a later publication.

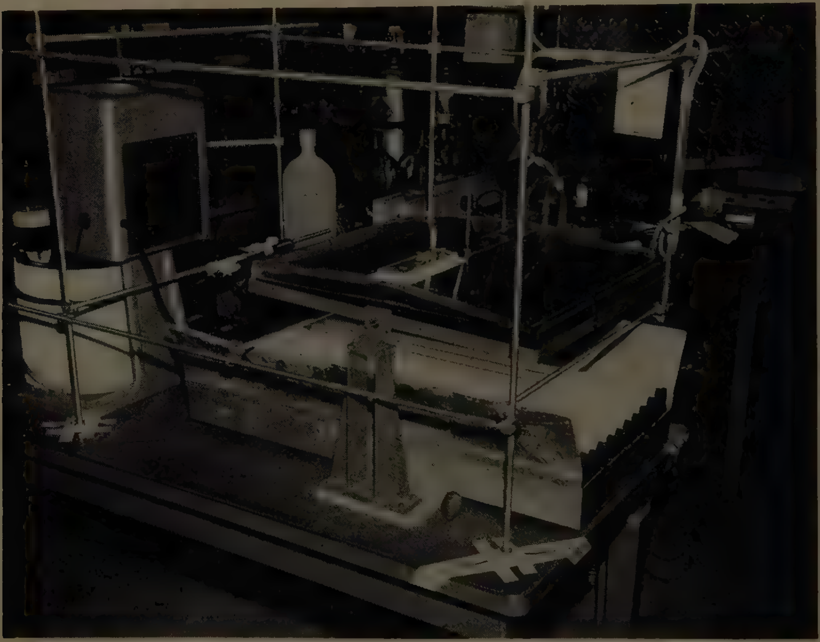

FIGURE 7. A photograph of the type of unit used for the mass culture of *Scenedesmus obliquus*. A 23-inch square Lucite container is held in a rocking frame with fluorescent lighting from below. The culture, whose volume is 5 l., is maintained at 25° C. by circulating cooling water from the bath at the left through channels in the base of the container.

Growth of Escherichia coli. Strain K-12 of *E. coli* has been grown in a variety of completely and partially deuterated media. All the nutrient solutions had the composition (gm./l.): NH_4Cl, 1.0; $MgSO_4$, 0.10; KH_2PO_4, 3.0; Na_2HPO_4, 3.2; NaCl, 0.50; $CaCl_2$, 0.002; $FeCl_3$, 0.002. The organic substrates were ordinary and fully deuterated glucose. We emphasize that by "fully deuterated" we refer to both exchangeable and nonexchangeable positions in the molecules. All of the cultures in H_2O were initially grown on agar slants of the appropriate composition. The D_2O-glucose cultures were obtained by serial subculture at 31° C. in a DH_G* system, according to the sequence 0 →

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* See TABLE 4 for an explanation of the coding.

50 → 99.7 per cent D_2O, and then allowed to grow for storage on DH_G and DD_G agar slants.

Growth-rate experiments were carried out by inoculating (with the appropriate organism) about 8 ml. of nutrient medium contained in a 120 mm. × 20 mm. (O.K.) test tube fitted with gas inlet and outlet tubes in a standard taper glass joint. The gas inlet tube did not project into the liquid, but served to maintain a flow of filtered air above the culture. The tubes were held fixed at an angle of 45° in a constant temperature, rotary, water-bath shaker. Samples were removed periodically for plate-counts of viable cells; turbidity measurements were made directly on the culture solution by measuring the 90° scattered green light in a Brice-Phoenix Universal Light Scattering Photometer. Aseptic conditions were maintained throughout.

Figures 8 and 9 illustrate the type of growth curves observed using ordinary and deuterio-glucose as substrates in H_2O and D_2O. In the systems at 31° C. the principal slowing of the growth rate is due to the change from H_2O to D_2O with a slight slowing of the growth rate due to the deuterio-glucose. At 37° C., however, substitution of deuterio-glucose for ordinary glucose in D_2O strongly depressed the growth rate and caused a pronounced lag phase. The general appearance of the cells in all of these various cultures was so similar that differences in the morphology of the cells are unlikely to have any effect on the comparison of light-scattering measurements.

Table 4 is a summary of data obtained at 31° C. and 37° C. with ordinary and deuterated glucose with H_2O and D_2O. In comparing the results obtained with the different media at 31° C. we notice for both live cells and total turbidity a marked slowing of the growth rate when converted from H_2O to D_2O and a lesser slowing effect due to the deuterated glucose. At 37° C., however, we notice that in terms of total cells present there is considerable improvement in growth in the two H_2O media and a marked improvement in the DH_G system. However, the completely deuterated bacteria grew more slowly at 37° than at 31° C., both in terms of viable cells and total cells, but particularly in terms of viable cells. The data thus indicate an increase in death rate at the higher temperature without a parallel increase in the division rate. It would appear then that at 37° C. the total substitution of deuterium for hydrogen strongly increases the instability of the cell as compared to the partly substituted cell.

These data indicate that one or more structural or reaction patterns in the bacteria become considerably altered at the higher temperature because of incorporation of deuterium into nonexchangeable positions. Calvin *et al.*[15] have speculated, from their data on the helix-coil transition of deuterium-exchanged polypeptides, that the ratio of concentrations of helices to random coils in a biological system can be markedly affected by deuteration. Substitution at nonexchangeable positions may also be important in this respect, and with the availability of fully deuterated microorganisms it is now possible to study the effect on nonexchangeable deuterium on the configuration of nucleic acids and other macromolecules.

Yeast and Mold Fungi

As previously reported, the yeast *Torulopsis utilis* has been grown in a completely deuterated system containing a D_2O extract obtained from deuterated

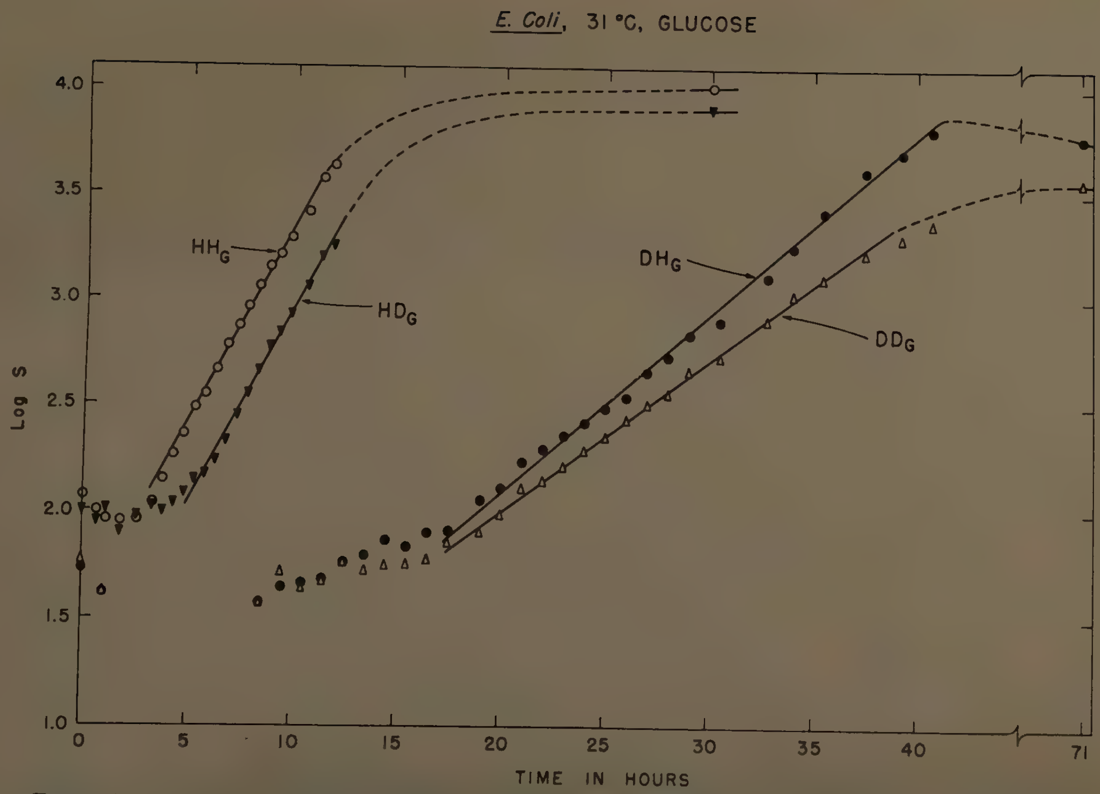

FIGURE 8. Growth curves for *Escherichia coli* (K-12) in glucose media at 31° C. "*S*" refers to the amount of light scattered at 90°, in arbitrary units. The effect of fully deuterated glucose on the growth rate appears minor when compared to the effect of changing from H_2O to D_2O. See TABLE 4 for an explanation of the coding.

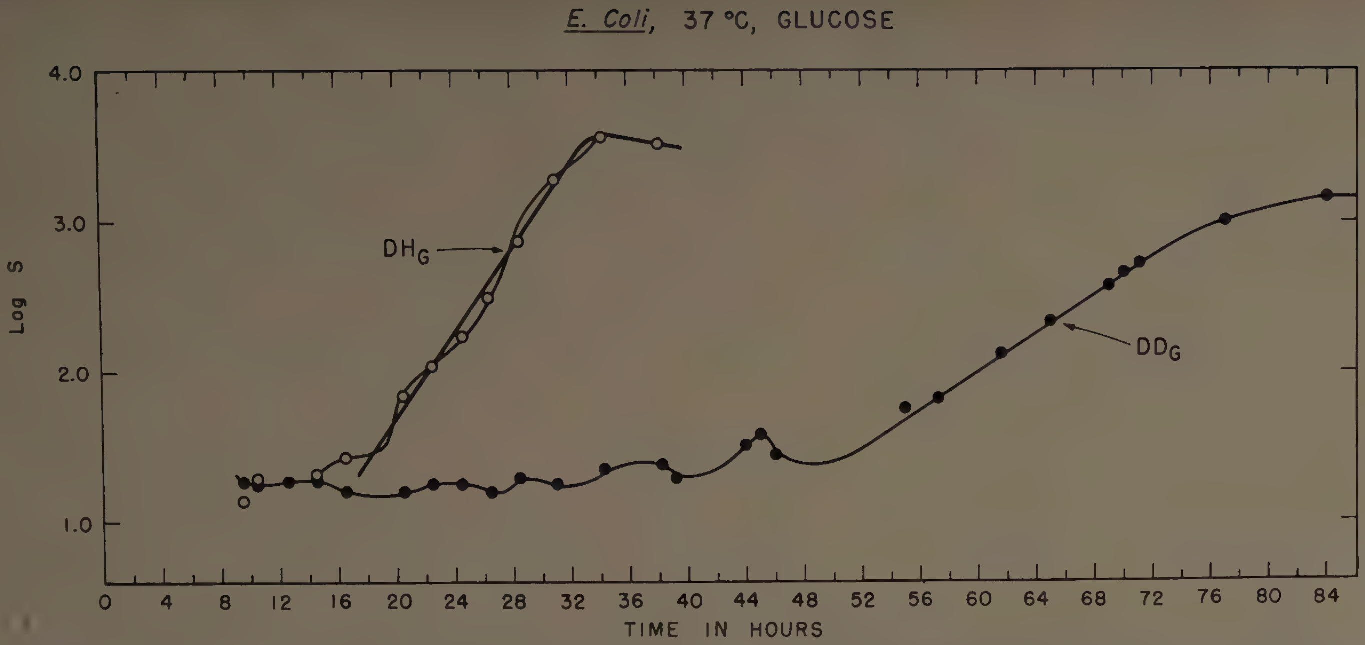

FIGURE 9. Growth curves for *Escherichia coli* (K-12) in D_2O at 37° C. "*S*" refers to the amount of light scattered at 90°, in arbitrary units. The substitution of fully deuterated glucose for ordinary glucose has markedly slowed the growth rate at this temperature.

algae. Ordinarily *T. utilis* grows well in H_2O on glucose and inorganic salts alone, but in 99.6 per cent D_2O this yeast apparently requires one or more additional nutritional factors. These can be supplied by an extract of deuterated algae. Once again the phenomenon of acid production upon inoculation of unadapted H_2O yeast into the fully deuterated medium was encountered. V. S. Mohan of this laboratory has studied the growth of *T. utilis* in D_2O in detail, and has found that the inhibitory effect of deuterium is overcome to a considerable extent by the addition of thiamine to the D_2O medium. A full account of these experiments will be published elsewhere.

A number of growth studies have been made with *Aspergillus niger** in fully and partially deuterated systems. The almost complete inhibition of the growth of this organism by 99.6 per cent D_2O in Czapek medium containing ordinary glucose has previously been reported.[16]

In the experiments to be reported here, cultures were grown in 50 to 60 ml. of Czapek medium in 125-ml. Erlenmeyer flasks placed on a rotary shaker. Various combinations of ordinary glucose and algae extract supplemented with deuterated glucose were used. The flasks were protected from the atmosphere, and a supply of filtered air was blown through each flask. One-tenth ml. of a light suspension of ordinary spores was used for inoculation. Under these growth conditions of the order of 100 mg. (dry wt.) of mycelium were obtained from an H_2O culture with ordinary glucose in one week.

TABLE 4

E. coli DOUBLING TIMES

Code	Medium water	Glucose	31° C.		37° C.	
			Live cells (hours)	Scattering (hours)	Live cells (hours)	Scattering (hours)
HH_G	H_2O	Ordinary	0.46	1.6	0.48	1.2
HD_G	H_2O	Deuterated	0.61	1.7	0.61	1.3
DH_G	D_2O	Ordinary	2.2	3.6	1.8	2.1
DD_G	D_2O	Deuterated	2.5	4.5	3.2	4.8

In a D_2O medium made up with ordinary glucose, growth is again extremely slow, and the yield after two weeks of growth is of the order of 1 mg. of mycelium. Hyphae in such cultures were larger than normal, segmented and branched, and contained large vacuoles. The addition of a mixture of vitamins consisting of thiamine, calcium pentothenate, nicotinic acid, biotin, pyridoxine, riboflavin, and vitamin B_{12} perhaps doubled the growth rate, but at the expense of creating larger and even more abnormal appearing hyphae. However, replacement of the ordinary glucose in the D_2O medium with 1 per cent deuterated algae extract yields a harvest of 30 to 40 mg. (dry wt.) of completely deuterated mycelium in two weeks. Algae extract, prepared by treating fully deuterated algae with boiling D_2O, contains by weight about 40 per cent sugar, principally sucrose. The hyphae of *Aspergillus* formed in this medium are similar in size and structure to those grown in ordinary water. A comparable but somewhat lower growth rate is obtained on 0.5 per cent algae extract fortified with 3 per cent deuterated glucose, and perhaps a somewhat higher growth rate on 0.5 per cent algae extract together with 3 per cent ordinary glucose. These latter experiments were carried out in 15-ml. cultures and the extrapolated values may be subject to some error. However, we conclude that the deuterated algae extract increases the growth rate of *Aspergillus* in a completely deuterated system to at least one sixth the rate in the ordinary hydrogen medium, and that further increases in growth rate in the deuterated media are probable.

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* General Biological Supply House, Chicago, Ill.

Protozoa

Paramecium caudatum, a ciliated protozoan, has been routinely maintained by us in 60 per cent D_2O cultures.[7] This protozoa was grown under nonsterile conditions in 100 ml. of lettuce infusion in cotton-stoppered 125-ml. Erlenmeyer flasks. The bacterial population was mixed. For all subcultures, 10 ml. transfers were used, except at the 60 per cent level of D_2O. This *Paramecium* was adapted to 60 per cent D_2O according to the following protocol: $0 \rightarrow 38 \rightarrow 44 \rightarrow 55 \rightarrow 60$ per cent D_2O. Fresh cultures were allowed to grow for 2 to 3 weeks before transfer to the next higher deuterium level. A semiquantitative measure of cell numbers was made by microscopic scanning 0.05 ml. of culture fluid.

At D_2O concentrations below 60 per cent, no major abnormalities in the cells or their growth were observable. However, after transfer into 60 per cent D_2O, an increase in cell numbers was observed in about two weeks, and at the same time, "monster" forms appeared. These monsters were shaped like a "dog-leg" similar to drawing "f" of Calkins.[17] During adaptation the number of monsters present was about 10 per cent of the total population, but after continuous culture at 60 per cent D_2O for a few months the monsters were no longer apparent.

A D_2O concentration of 60 per cent seemed to represent a natural barrier, as it was very difficult to sustain growth at higher D_2O levels. Growth would take place as the D_2O concentration was raised to 65 per cent, and even briefly at 70 per cent by adding 0.5 ml. of 99.6 per cent D_2O lettuce infusion to the cultures every other day. When additions of fresh lettuce infusion were stopped, the cultures soon died. Some difficulty was encountered in maintaining the 60 per cent D_2O stock cultures by the usual procedure (10 ml. inoculation into 90 ml. of fresh lettuce infusion). Instead, when these cultures reached their maximum concentration of 200 to 250 cells per ml., the cultures were divided into two equal 50 ml. portions, and 50 ml. of fresh lettuce infusion was added to each. This procedure was repeated every two weeks for 5 to 6 months before the experiment was terminated. In these 60 per cent D_2O cultures *P. caudatum* was shorter and thicker than its H_2O counterpart; its movements were somewhat sluggish and less graceful than in normal animals. Otherwise, this deuterated protozoan was quite similar in structure to the normal organism, but a detailed study has not yet been made.

Paramecium busaria was grown under conditions similar to those employed for *P. caudatum*. The organism was inoculated into 30 per cent D_2O lettuce infusion from an H_2O culture, and grew after some delay. Serial subculture with increments of 5 per cent D_2O adapted this species to growth in 45 per cent D_2O, and then slowly to 50 per cent D_2O. At this D_2O concentration "monster" forms again appeared, but these disappeared after repeated subculture at the 50 per cent level. Attempts to increase the D_2O concentration at which growth occurred were not successful.

Summary

Data has been presented on the culture of a variety of algae and other microorganisms in highly deuterated systems. Growth rates for the unicellular green algae *Scenedesmus obliquus* and *Chlorella vulgaris* in 99.6 per cent D_2O have been improved so that they approach one half the rates in H_2O nutrient media. *S. obliquus* has also been successfully mass cultured in five-liter units. The blue-green alga *Plectonema calothricoides* has been grown on a continuous laboratory scale in 99.6 per cent D_2O. A number of other green, blue-green, and red algae, and diatoms have been cultured at D_2O levels from 75 to 99.6 per cent. The bacterium *Escherichia coli* (K-12), the yeast *Torulopsis utilis*, and the mold fungus *Aspergillus niger* were grown in fully deuterated media. Protozoa have been cultured at somewhat lower D_2O concentrations: *Paramecium caudatum* routinely in 60 per cent D_2O lettuce infusion and *Paramecium bursaria* in 50 per cent lettuce infusion. In general, algae are best adapted to D_2O by serial subculture, 0 to 75 to 99 to 99.6 per cent D_2O, but *Chlorella*, *E. coli*, *T. utilis*, and *A. niger* can be grown by direct transfer from H_2O to 99.6 per cent D_2O nutrient media.

Acknowledgments

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OBSERVATIONS ON THE INFLUENCE OF DEUTERIUM ON BACTERIAL GROWTH*

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Introduction

When Harold C. Urey announced the discovery of deuterium in 1932,[1] he suggested that the replacement of hydrogen by deuterium might give rise to significant changes in various biological systems. This supposition promptly was confirmed by the work of Lewis,[2] which showed that the substitution of hydrogen by deuterium in higher plants and animals was, in general, a toxic process. However, limitations in the supply of available deuterium handicapped the early investigations of this phenomenon. Thus, the early data which have been summarized by Morowitz and Brown[3] suggested that, while deuterium was less toxic for unicellular forms of life than for higher plants and animals, there were marked individual variations in this respect.

In general the early studies on the influence of deuterium on bacterial growth dealt with low concentrations of D_2O, and the results were not uniform. However, Itoh *et al.* in 1935[4] reported that the growth of *Mycobacterium tuberculosis* was stimulated by low concentrations of deuterium ranging from 0.03 to 2.2 per cent. Harvey and Taylor[5] studied the effects of deuterium in concentrations ranging from 36 to 86 per cent D_2O on *Vibrio phosphorescens* and found that growth was reduced at the higher concentrations. On the other hand, Hansen and Blegen[6] reported that the growth of *Staphylococcus albus* and *Eberthella typhosa* was not inhibited by concentrations as high as 92 per cent D_2O.

The present availability of relatively large quantities of deuterium has made possible more exact and comprehensive studies on the biological effects of deuterium in various concentrations. This report summarizes in detail our studies conducted on various microbial species utilizing growth curves obtained under standardized laboratory conditions as a means of demonstrating these effects of deuterium.

Methods and Materials

The effects of various concentrations of deuterium in appropriate media were evaluated using pure cultures of the following microorganisms:

(1) Mycobacteria.

(*a*) *M. tuberculosis* (var. hominis). Four strains were studied which had been isolated from patients of the Suburban Cook County Tuberculosis Hospital-Sanitarium: 9450—isoniazid sensitive, catalase positive; 3696—isoniazid sensitive, catalase positive; 321—isoniazid resistant, catalase negative; and 51—isoniazid sensitive, catalase positive.

(*b*) Unclassified strain (rapid-grower): 2801—isoniazid and streptomycin resistant.

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(2) Group C hemolytic streptococci.
(3) Type I pneumococci.
(4) *Escherichia coli.*
(5) *Candida albicans.*

Appropriate liquid media for these various microorganisms were prepared in concentrated solutions and diluted with deuterium oxide to the desired deuterium concentrations. All concentrations were checked at the beginning and end of each experiment by the spectrophotometric technique method of Katz *et al.*[7]

Mycobacteria. The concentrations of deuterium used in these studies were 25, 50, 75, and 100 atom per cent, except in one experiment where levels of 50, 60, 70, 80, 90, and 100 atom per cent D were employed. These concentrations were made by diluting double strength Dubos broth base suspended in D_2O and further adjusted to the final strength by either D_2O or H_2O. Dubos broth containing no deuterium was used as the control medium in each experiment.

The mycobacterial cultures were grown in Difco Bacto Dubos broth for 14 days before inoculation into the deuterium-containing medium. A volume of 0.1 ml. of this suspension was inoculated into 10 ml. of deuterium-containing Dubos broth in 200 × 25 screw cap test tubes. The screw caps were lined with aluminum foil in order to diminish the loss of deuterium.

Prior to inoculation, 5.0 ml. of each deuterium concentration was pipetted into another set of identical screw cap test tubes. These were incubated under the same conditions as the inoculated set except that at the beginning and end of each experiment, 1.0-ml. samples were removed from each tube. These samples then were analyzed quantitatively for deuterium content by means of the technique previously cited.[7]

Appropriate samples of the contents of each inoculated tube were taken at the beginning of each experiment and at various time intervals during the period of incubation. These samples were plated quantitatively in the following manner. At the time of sampling, a volume of 0.5 ml. was removed from each tube and serial dilutions were made, using 4.5-ml. blanks of deuterium-free Dubos broth. Aliquots of 1.0-ml. volume of the appropriate dilutions were spread on the surface of modified oleic acid albumin agar plates.* The

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* Modified oleic acid albumin agar:

Mineral base

KH_2PO_4	10.00 gm.
$Na_2HPO_4 \cdot 7\ H_2O$	62.50 gm.
$Na_3C_6H_5O_7 \cdot 2\ H_2O$	15.00 gm.
$MgSO_4 \cdot 7\ H_2O$	6.00 gm.
$C_6H_5O_7Fe \cdot C_6H_5O_7(NH_4)_2H$	0.05 gm.
Distilled water	q.s. 500 ml.

Oleic acid—albumin complex

5 per cent solution of bovine albumin (Fraction V) made in 2 per cent NaCl.
Neutralize with 1 N NaOH; Seitz filter.
Heat in water bath at 56° C. for 30 min.; Seitz filter.
Dissolve 0.12 ml. oleic acid in 10 ml. of $\frac{1}{20}$ N NaOH by shaking with a rotary motion in a small flask.
Add 5 ml. of oleic acid solution to 95 ml. of 5 per cent albumin; Seitz filter.
Incubate over night to check sterility.

Complete solid medium

Mineral base	9.0 ml.

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plates then were incubated at 37° C. for 28 days, and the resultant colonies counted using a dissecting microscope. The results of these plate counts were used to determine the bacterial population per milliliter of medium.

Group C hemolytic streptococci. A 0.1-ml. volume of stock culture of Group C hemolytic streptococci was inoculated into 10 ml. of Difco Bacto heart infusion broth containing 5 per cent sheep cells and incubated at 37° C. for 24 hours. This procedure was repeated twice, and the resultant culture then was used to inoculate all tubes of the control and deuterium series in a volume of 0.1 ml. of stock culture to each 10 ml. of medium.

The deuterium was incorporated into Difco Bacto heart infusion broth or tryptose phosphate broth in desired concentration by suspending the dehydrated medium in appropriate mixtures of D_2O and H_2O. The liquid medium then was tubed in 10-ml. volumes, inoculated with culture, and incubated at 37° C.

At appropriate time intervals serial dilutions from the control and deuterium series were made, utilizing pour plates of Difco Bacto blood agar base containing 5 per cent sheep blood cells using 0.5-ml. aliquots at each dilution. The plates were incubated at 37° C. for 48 hours and the number of colonies counted. The results of these plate counts were used to determine the bacterial population per milliliter of medium.

Type I pneumococci. The deuterium was incorporated into Difco Bacto tryptose phosphate broth. Poor growth resulted from the incubation of pneumococci in these media. To correct this, one experiment was done in which 5 per cent lyophilized rabbit blood, reconstituted with D_2O, was added to the tryptose phosphate broth. The other materials and methods were the same as described above for the Group C hemolytic streptococci.

Escherichia coli. Initially the stock culture was grown in heart infusion broth at 37° C. for 24 hours. A volume of 0.1 ml. of the resultant culture then was used to inoculate each 10 ml. of the control and deuterium series. The deuterium was incorporated in the desired concentrations in tryptose phosphate broth. All other details of procedure were the same as for the Group C hemolytic streptococci.

Candida albicans. Difco Bacto Sabouraud's dextose liquid medium was used for all studies on this organism. Pour plates for sampling were made from 0.5-ml. aliquots of appropriate serial dilutions using Difco Bacto Sabouraud's dextrose agar. The desired concentrations of deuterium were obtained by using D_2O and H_2O in varying proportions to prepare the Sabouraud's dextrose liquid medium.

Results and Duscussion

Mycobacteria. The four strains of *M. tuberculosis* (typical, human type) selected for this study were representative cultures which had been recently isolated from patients with active pulmonary tuberculosis. Three of the strains

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N-Z Amine, Type B		0.18	gm.
Distilled water	q.s.	190	ml.
Agar		3.0	gm.

Autoclave at 15 lb. for 15 min.
Cool to 48° C.; add 10 ml. oleic acid albumin complex.
Mix well and pour into Petri dishes.

were isoniazid sensitive and catalase positive (9450, 3696, and 51), and the fourth culture was isoniazid resistant and catalase negative (321).* This last strain was included as a means of determining if the loss of catalase activity and acquisition of isoniazid resistance would, in any way, alter the response of tubercle bacilli to the toxic effect of high concentrations of deuterium.

The data obtained from the cultivation of strain 9450 in various concentrations of deuterium over a 49-day period are summarized in FIGURE 1. It reveals that inhibition of growth attributable to deuterium toxicity occurs only at very high concentrations (95 atom per cent) and is not clearly seen until after the seventh day. In addition, there is the suggestion that, after the first 21 days of growth, the intermediate concentrations of deuterium (from 25 to 75 atom per cent) may have a slightly stimulatory effect. The results of the quantitative analyses for deuterium performed on specimens obtained at the beginning and conclusion of the 49-day experiment were reassuring in that, in no instance was the final deuterium concentration greatly at variance from the original level set up in each series.

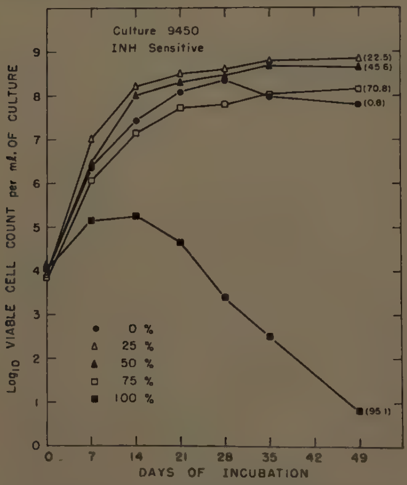

FIGURE 1. Growth of *M. tuberculosis* (culture 9450), an isoniazid-sensitive strain, in Dubos medium containing various concentrations of deuterium.

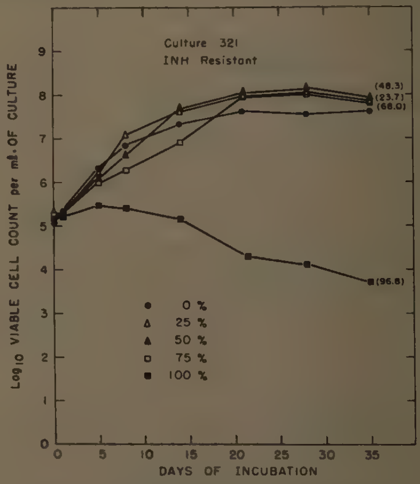

FIGURE 2. Growth of *M. tuberculosis* (culture 321), an isoniazid-resistant strain, in Dubos medium containing various concentrations of deuterium.

The results of experiments with the other two isoniazid-sensitive, catalase-positive strains of human type tubercle bacilli (3696 and 51) are summarized in TABLES 1 to 3. The general pattern of response to higher deuterium concentrations is the same as that of 9450. In these three experiments growth of tubercle bacilli was equal to or greater than that of the control series until concentrations above 80 atom per cent of deuterium were attained. However, when the deuterium concentration exceeded 80 atom per cent, growth was significantly inhibited.

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* Cultures of this strain showed growth equal to that of the control on media containing 5.0 γ of isoniazid per milliliter of media.

A series of experiments were done on culture 321, the isoniazid-resistant, catalase-negative strain. These results are summarized in FIGURE 2 and do not differ in any significant degree from the response of isoniazid-sensitive, catalase-positive strains of tubercle bacilli to the range of deuterium concentrations tested. The data obtained failed to indicate any difference in sensitivity to high deuterium concentrations between isoniazid-sensitive and resistant tubercle bacilli.

TABLE 1

GROWTH OF *M. TUBERCULOSIS* (CULTURE NO. 3696) IN VARIOUS CONCENTRATIONS OF DEUTERIUM AS DETERMINED BY BACTERIAL POPULATION PER MILLILITER OF DUBOS MEDIUM

Deuterium concentration (atom per cent)		Days of incubation					
Theoretical	Actual*	0	7	14	21	28	35
100	97.0/88.1	3.1×10^5	1.9×10^6	6.9×10^6	8.2×10^6	2.5×10^7	5.8×10^7
75	71.6/64.9	3.2×10^5	6.6×10^6	4.2×10^7	8.7×10^7	1.7×10^8	2.2×10^8
50	46.3/42.8	2.5×10^5	2.3×10^7	1.1×10^8	2.2×10^8	2.4×10^8	2.8×10^8
25	22.8/23.5	2.8×10^5	2.2×10^7	8.4×10^7	1.7×10^8	2.4×10^8	2.8×10^8
0	1.5/0	3.9×10^5	6.4×10^6	3.6×10^7	7.6×10^7	1.2×10^8	1.2×10^8

* Numerator—deuterium concentration (atom per cent) determined quantitatively at start of experiment. Denominator—deuterium concentration (atom per cent) determined quantitatively at end of experiment.

TABLE 2

GROWTH OF *M. TUBERCULOSIS* (CULTURE NO. 51) IN VARIOUS CONCENTRATIONS OF DEUTERIUM AS DETERMINED BY BACTERIAL POPULATION PER MILLILITER OF DUBOS MEDIUM

Theoretical deuterium concentration (atom per cent)	Days of incubation									
	0	1	2	3	6	9	14	21	28	35
100	5.0×10^6	6.2×10^6	6.4×10^6	8.5×10^6	7.7×10^6	4.7×10^6	3.2×10^6	1.7×10^6	1.7×10^6	4.5×10^5
75	5.5×10^6	6.1×10^6	9.2×10^6	7.8×10^6	7.4×10^6	1.1×10^7	4.6×10^7	1.6×10^8	2.9×10^8	2.6×10^8
50	5.0×10^6	5.5×10^6	8.6×10^6	1.1×10^7	2.1×10^7	5.7×10^7	2.0×10^8	2.7×10^8	4.2×10^8	3.3×10^8
25	5.2×10^6	7.0×10^6	9.8×10^6	1.1×10^7	3.0×10^7	7.1×10^7	1.2×10^8	1.6×10^8	2.4×10^8	1.8×10^8
0	6.1×10^6	7.6×10^6	—	8.5×10^6	1.8×10^7	3.1×10^7	9.7×10^7	9.2×10^7	1.4×10^8	1.1×10^8

In all of the experiments with tubercle bacilli, summarized in FIGURES 1 and 2, and TABLES 1 to 3, deuterium concentrations between 25 and 70 atom per cent were found to have a slightly stimulatory effect upon the growth rate. However, in all cases, deuterium concentrations in excess of 80 atom per cent produced less growth than in the control cultures. Above this level the toxicity of deuterium increased in a linear fashion as the concentration approached 100 atom per cent.

It must be pointed out that the quantitative procedure used for the determination of these growth curves for cultures of tubercle bacilli presents many inherent difficulties which make detailed analysis of the data unwarranted. The results obtained probably are significant only insofar as they demonstrate significant inhibition at the highest deuterium concentrations in the culture media (greater than 85 atom per cent). Further and more precise studies would be required before the stimulatory effect of the intermediate concentrations of deuterium (25 to 75 atom per cent) can be considered a significant phenomenon. The results at the intermediate deuterium concentrations could reflect physical changes in the cording or aggregation of the tubercle bacilli in the liquid cultures. Thus, an apparent increase in the number of colony-producing particles would not necessarily imply greater growth but could reflect increased dispersion of the cellular components in the media. This possibility has not been evaluated in these experiments.

TABLE 3

GROWTH OF *M. TUBERCULOSIS* (CULTURE NO. 51) IN INTERMEDIATE CONCENTRATIONS OF DEUTERIUM AS DETERMINED BY BACTERIAL POPULATION PER MILLILITER OF DUBOS MEDIUM

Deuterium concentration (atom per cent)		Days of incubation							
Theoretical	Actual*	0	1	3	7	14	21	28	35
100	92.4/68.8	4.4×10^4	1.5×10^5	3.5×10^5	3.7×10^5	4.1×10^5	2.0×10^5	1.7×10^5	2.5×10^5
90	84.0/82.5	5.2×10^4	1.0×10^5	2.5×10^5	5.3×10^5	1.2×10^6	3.1×10^6	7.1×10^6	2.0×10^7
80	74.0/59.0	6.5×10^4	1.1×10^5	3.6×10^5	6.8×10^5	2.8×10^6	1.9×10^7	4.9×10^7	8.5×10^7
70	67.6/61.6	7.0×10^4	1.6×10^5	3.6×10^5	1.1×10^6	6.7×10^6	7.9×10^7	1.7×10^8	1.8×10^8
60	57.0/54.6	5.9×10^4	1.2×10^5	1.2×10^6	1.7×10^6	5.8×10^7	1.4×10^8	2.8×10^8	3.4×10^8
50	49.1/43.6	5.4×10^4	3.0×10^5	1.0×10^6	3.8×10^6	1.4×10^8	1.5×10^8	2.2×10^8	3.5×10^8
0	—	6.2×10^4	2.6×10^5	1.5×10^6	5.5×10^6	7.1×10^7	1.2×10^8	1.7×10^8	1.5×10^8

* Numerator—deuterium concentration (atom per cent) determined quantitatively at start of experiment. Denominator—deuterium concentration (atom per cent) determined quantitatively at end of experiment.

The effect of various concentrations of deuterium on the growth of strain 2801, in general, was the same as that shown by typical human type tubercle bacilli. The results of this experiment are summarized in FIGURE 3. This culture was characterized as a "rapid-grower," and was highly resistant to both isoniazid and streptomycin.* Analysis of FIGURE 3 shows that inhibition of growth was apparent with increasing concentrations of deuterium and was most evident at 95 atom per cent. The intermediate concentrations of deuterium failed to show any definite stimulatory effect on the growth of culture 2801 and, in fact, at 50 and 75 atom per cent, growth was less than was observed in the control. This observation suggests the possibility that the "rapid-grower" strain may be somewhat more sensitive to the toxic effects of the replacement of hydrogen by deuterium than are human-type tubercle bacilli. However, additional experiments would be needed before this point could be considered proven.

Group C hemolytic streptococci. The results of a typical experiment evaluating the influence of various concentrations of deuterium on the growth of Group C hemolytic streptococci in heart infusion broth are summarized in FIGURE 4. This experiment uniformly demonstrated increasing inhibition of growth with ascending concentrations of deuterium. This response was characteristic of all experiments dealing with group C hemolytic streptococci in which an adequate medium was provided. However, as shown in FIGURE 5, the growth of Group C hemolytic streptococci on an inadequate substrate at various concentrations of deuterium presents a more complex picture. It can be seen that during the early period of adjustment to the meager medium, the presence of deuterium in high concentrations (from 70 to 95 atom per cent) diminishes the initial rate of death. However after 24 hours, growth becomes established and is as rapid in the deuterium-containing media as in the control series. Subsequent to 48 hours, exhaustion of the media develops, and the presence of deuterium is associated with demonstrably increased toxicity. These results suggest that the biological effects of deuterium are exerted in different ways at various stages of growth within a culture. However, in terms of the data presented, little more can be said as to the mechanisms by which the toxic effect of deuterium becomes apparent. Certainly, a careful analysis of the growth curves plotted in FIGURE 5 does not support the suggestion that the inhibitory influence of high concentrations of deuterium becomes apparent only at a time of bacterial multiplication.

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* Growth equal to control at concentrations of 5 γ of isoniazid and 100 γ of streptomycin per milliliter of media respectively.

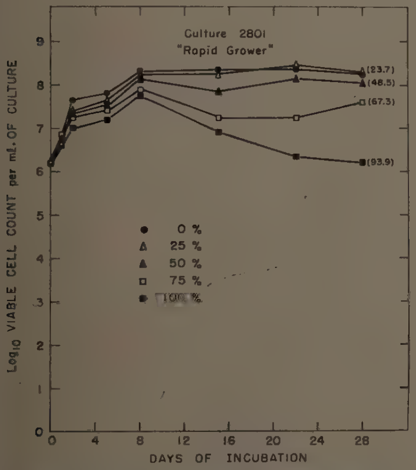

FIGURE 3. Growth of culture 2801, a "rapid-grower" atypical mycobacterial strain, in Dubos medium containing various concentrations of deuterium.

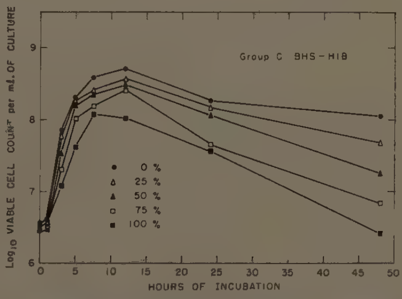

FIGURE 4. Growth of Group C hemolytic streptococci in heart infusion broth containing various concentrations of deuterium.

Type I pneumococci. As was expected, the growth of pneumococci in tryptose phosphate medium was unsatisfactory, and the results of a typical experiment are shown in FIGURE 6. The tryptose phosphate medium had been used because it was an easy one in which to incorporate deuterium at the desired levels of concentration. The data summarized in FIGURE 6, however, were quite consistent in demonstrating a decreasing rate of death as the deuterium concentration was elevated. Thus, the deuterium-free control culture was sterile by the 24th hour of incubation while subcultures from the 100 atom per cent deuterium culture were still positive at the 48th hour. The intermediate concentrations of deuterium gave survival curves falling between these extremes.

A second experiment was performed analyzing the survival of pneumococci in tryptose phosphate medium containing different concentrations of deuterium. This experiment was initiated with a larger bacterial inoculum of Type I pneumococci and was extended over a longer period of time; it is summarized in FIGURE 7. It can be seen that, during the first 24 hours, the results closely duplicated those shown in FIGURE 6. However, with the further passage of time, significant bacterial growth occurred at the 50 to 100 atom per cent concentrations of deuterium. The control and 25 per cent deuterium cultures failed to show viability at 72 hours, whereas the 75 and 100 per cent deuterium cultures had returned to approximately their original population levels at 120 hours. These results once again demonstrated that deuterium in high concentrations diminished the initial rate of death of pneumococci in an adverse medium. In addition, however, these results also suggested that the surviving organisms in the higher deuterium concentrations were able to develop adaptive mechanisms which permitted growth to be initiated. It is possible that this growth, which became apparent in the higher deuterium concentrations after 24 hours incubation, may only reflect the survival of greater numbers of pneumococci up to the time period in question. Thus, the populations of pneumococci surviving the first 24 hours at concentrations of 50 to 100 per cent deu-

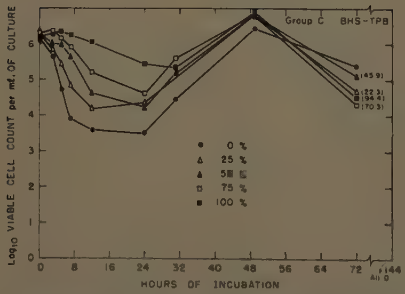

FIGURE 5. Growth of Group C hemolytic streptococci in tryptose phosphate broth containing various concentrations of deuterium.

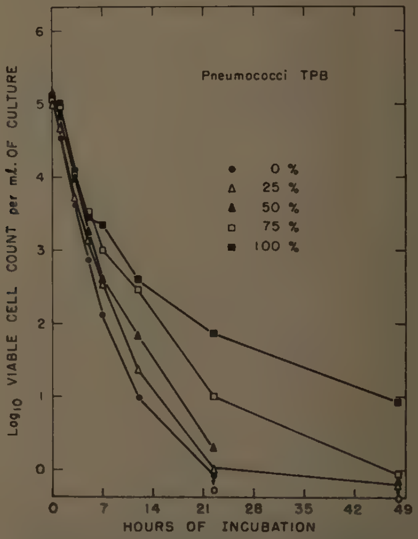

FIGURE 6. Growth of Type I pneumococci in tryptose phosphate broth containing various concentrations of deuterium.

terium were from 10 to 100 times greater than were present in the control and 25 per cent deuterium series.

The growth curves summarized in FIGURE 8 are those obtained from an experiment in which Type I pneumococci were grown in tryptose phosphate media enriched by the addition of 5 per cent lyophilized rabbit blood cells. This was a much more adequate medium for the growth of pneumococci. The data reveal that, under these conditions, increasing deuterium concentrations prolong the initial lag phase of growth during the first 12 hours of incubation and, thereafter, delay the time at which peak growth is manifest. Subsequent decline in population is slowest in the higher deuterium concentrations. Thus, the control and 25 per cent deuterium series revealed no viable pneumococci after 48 hours; whereas, the 100 per cent deuterium series still had a few pneumococci surviving at 96 hours. These results suggest a definite protective effect of deuterium in concentrations above 50 atom per cent in this medium on pneumococci although, once again, this phenomenon is not uniform and is modified in relation to the phase of growth of the culture.

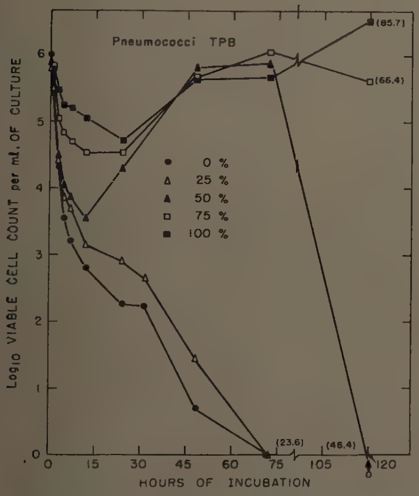

FIGURE 7. Growth of Type I pneumococci in tryptose phosphate broth containing various concentrations of deuterium.

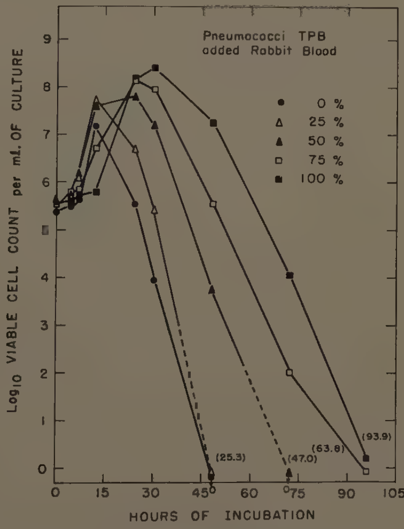

FIGURE 8. Growth of Type I pneumococci at various concentrations of deuterium contained in tryptose phosphate broth with added rabbit blood cells.

Escherichia coli. The growth of *E. coli* in tryptose phosphate broth was inhibited progressively at increasing concentrations of deuterium. The results of a typical experiment are summarized in FIGURE 9 which demonstrates the uniformity of this inhibition.

Candida albicans. Cultures of this organism were found to be relatively insensitive to variations in deuterium concentration. FIGURE 10 summarizes a typical experiment in which cultures of *Candida albicans* were grown in Sabouraud's dextrose liquid medium at different concentrations of deuterium.

The specific growth curves obtained reveal that during the early period of incubation, up to less than 96 hours, deuterium in increasing concentrations exerts a slight inhibitory action. However, after 100 hours, survival at the intermediate concentrations of deuterium is somewhat better than that seen in the control series. These differences, however, are small, and their significance is doubtful.

It should be emphasized that the studies cited in this report are only, in a sense, descriptive, and they do not permit an analysis of the precise biochemical mechanisms which are involved in the replacement of hydrogen by deuterium. The data obtained indicate that such replacement, if carried to extremes, uniformly will inhibit bacterial growth. However, they throw little light on the manner in which this result is obtained. Furthermore, different bacterial species react in various ways to the intermediate concentrations of deuterium. These species differences undoubtedly depend upon many factors, of which mean generation time, specific enzyme pathways, and respiratory characteristics are but a few. It seems clear, however, that studies with deuterated media offer future promise as tools for further investigating the mechanisms and dynamics of cellular growth.

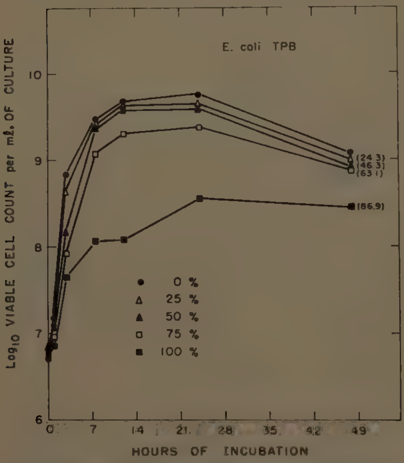

Figure 9. Growth of *E. coli* in tryptose phosphate broth containing various concentrations of deuterium.

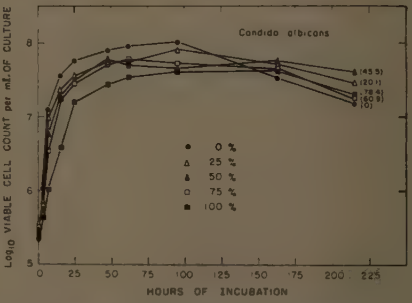

Figure 10. Growth of *Candida albicans* in Sabouraud's dextrose liquid medium containing various concentrations of deuterium.

Summary

The influence of different concentrations of deuterium in liquid media on the growth of various microbial species was studied. The concentrations of deuterium studied ranged from 0 to 95 atom per cent.

(1) Cultures of *M. tuberculosis* showed inhibition of growth only when the deuterium concentration surpassed 80 atom per cent. Deuterium concentrations between 25 and 80 atom per cent appeared to stimulate growth slightly but this effect was not apparent until after 21 days incubation. Cultures of

an unclassified "rapid-grower" strain of acid-fast bacilli showed only progressive inhibition of growth with increasing concentrations of D_2O.

(2) On adequate media, cultures of Group C hemolytic streptococci demonstrated an inverse relationship between growth and deuterium concentration. On adverse media, the initial decline in bacterial population was inhibited progressively by increasing concentrations of deuterium, but after growth became established deuterium toxicity again became evident.

(3) Cultures of Type I pneumococci under poor conditions of growth showed evidence of a protective action of the higher concentrations of deuterium against the initial decline in bacterial population. Once growth became established it reached higher levels and persisted longer as the deuterium concentration was increased.

(4) The growth of *E. coli* was found to be progressively inhibited by increasing deuterium concentrations.

(5) Cultures of *Candida albicans* showed little sensitivity to increasing concentrations of deuterium.

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THE MUTAGENIC EFFECT OF D_2O ON BACTERIOPHAGE T4*

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The study of the genetic fine structure of T4 bacteriophage (Benzer, 1955) and of the effectiveness of various mutagens in causing forward and reverse mutations in this phage (Litman and Pardee, 1956; DeMars, 1953; Benzer and Freese, 1958; Brenner *et al.*, 1958; Freese, 1959) has held out the hope for an understanding of the mechanism of mutation. In this connection it has become increasingly desirable to examine the mutagenic effect of especially those agents whose action is most easily susceptible to a direct chemical interpretation. The present work concerns an investigation of the mutagenic effect on phage T4 of deuterium oxide, which has previously been shown to be mutagenic for the bacterial host of this virus (DeGiovanni and Zamenhof, 1959), and whose mutagenic action should derive from isotope effects on the constituents of the replicating DNA.

Methods

Growth of T4 phage in D_2O. An aliquot of a culture of *Escherichia coli* strain BS, grown overnight in modified MI medium‡ (100 per cent H_2O) to a final density of 10^9 cells per ml., was inoculated into aliquots of fresh MI medium (containing various concentrations of D_2O) to a density of 5×10^6 cells per ml. The culture was agitated at 37° C., until a bacterial titer of 5×10^7 cells per ml. had been reached. At this point, the culture was infected with 10^3 T4 phages per ml. and *l*-tryptophan added to a final concentration of 10 μg. ml. Each infected culture was divided into 10 aliquots of 2 ml. each and agitated at 37° C. for 18 hours. Lysis of the infected culture was then induced by chloroform.

For measuring the forward mutation to the *r* plaque type, the phage inoculum was the wild type of strain T4D (Doerman and Hill, 1953). The progeny phage were assayed on nutrient agar plates seeded with strain BS, on which all *r* mutants exhibit the *r* phenotype. For measuring the reverse mutation from the *rII* mutant to the rII^+ wild type, various *rII* mutants of strain T4B (Benzer, 1955) were used as the phage inoculum. The progeny phage were assayed on plates seeded with strain K12 112, on which only rII^+ revertants can form plaques (Benzer, 1955) and on plates seeded with strain BS on which both rII^+ revertants and *rII* mutants form plaques.

Experimental

Growth of E. coli bacteria and T4 phage in D_2O. TABLE 1 presents the results of experiments in which *E. coli* bacteria and T4 phage were grown in MI media

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‡ Modified MI medium contains per liter H_2O: 7 gm. Na_2HPO_4, 3 gm. KH_2PO_4, 1 g NH_4Cl, 1 ml. 1 *M* $MgSO_4$, 2 ml. 25 per cent NaCl, 10 ml. 10 per cent glucose, 0.2 ml. 0.5 *M* $CaCl_2$, and 0.3 ml. 0.01 *M* $FeCl_3$.

containing various concentrations of D_2O. It is evident that the final phage titer attainable and the growth rate of the bacteria decreases sharply when the concentration of D_2O exceeds 50 per cent. Thus at these concentrations deuterium appears to affect adversely the physiology of growth of bacterial virus and bacterial cell.

Forward mutation. Preliminary experiments indicated that no very obvious increase in the proportion of T4 mutants appeared at any of the D_2O concentrations shown in TABLE 1. A more thorough investigation was, however, carried out to test the mutagenic effect of 50 per cent D_2O. In this experiment, T4 wild type phages were grown in 20 parallel cultures, of which half contained 50 per cent D_2O and half 0 per cent D_2O and the number of *r* mutants scored among the progeny. The result of this experiment was that in 0 per cent D_2O and 50 per cent D_2O the median values for the number of *r* mutants per 1000 r^+ phage were 2.1 and 0.9 respectively. The median total phage titer for the 0 per cent D_2O cultures was 2.1×10^{10} per ml., while the median titer for the 50 per cent D_2O cultures was 1.6×10^{10} per ml. Under the conditions of this experiment, the decrease in the mutant frequency from 2.1 in the H_2O cultures to 0.9 in the 50 per cent D_2O cultures cannot be considered significant. In contrast, a median value of 37 *r* mutants per 1000 phage was observed in five parallel cultures to which 200 μg. per ml. of the mutagen 5-bromodeoxyuridine (Litman and Pardee, 1956) had been added at the time of inoculum with T4 wild type phage. A concentration of 50 per cent D_2O therefore does not seem to exert any detectable effect on the forward mutation T4$r^+ \rightarrow$ T4*r*.

TABLE 1

THE DIVISION TIME OF *ESCHERICHIA COLI*, STRAIN BS, AND THE FINAL T4 PHAGE TITER ATTAINABLE IN MODIFIED MI MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF D_2O

Concentration of D_2O in medium (%)	Division time of bacteria at 37° C. (hours)	Final phage titer*
0	1.45	12×10^9 phage/ml.
40	—	4.3×10^9
50	1.75	—
60	—	3.4×10^9
80	—	0.8×10^9
95	3.0	0.5×10^9

* Median of 5 parallel cultures grown from an initial inoculum of 10^3 phages per ml.

Reverse mutation. The measurement of the reversion rate of *rII* mutants to the wild type is a much more sensitive and meaningful index of the mutagenic effect of an agent than the measurement of the forward mutation rate from the wild r^+ to the *r* phenotype (Freese, 1959). While in a measurement of the forward rate one must distinguish between the wild type r^+ plaque morphology and a more or less continuous spectrum of mutant plaque types, only two phenotypes are involved in a measurement of the reverse mutation rate, the original *rII* mutant and the r^+ revertant: *rII* mutants do not form plaques on *E. coli* strain K12 112, while the r^+ revertants do. Any mutagenic agent, furthermore, may differ greatly in its ability to increase the reversion rate of different mutants. The pattern of mutants revertible by a particular mutagen provides then a characterization of the mutagenic agent (Freese, 1959). FIGURE 1 presents the result of an experiment in which the reversion index* of the *rII* mutant S-2-16-3 was determined at various D_2O concentrations. It is apparent that at progressively greater D_2O concentrations a significant increase in the reversion index occurs, which approaches its maximum value at 50 per cent D_2O; a further increase in D_2O concentration up to 90 per cent D_2O produces little augmentation of the reversion index. It thus appears that D_2O is mutagenic in this system, since the reversion index can be increased by a factor of more than ten over the spontaneous background reversion index in H_2O.

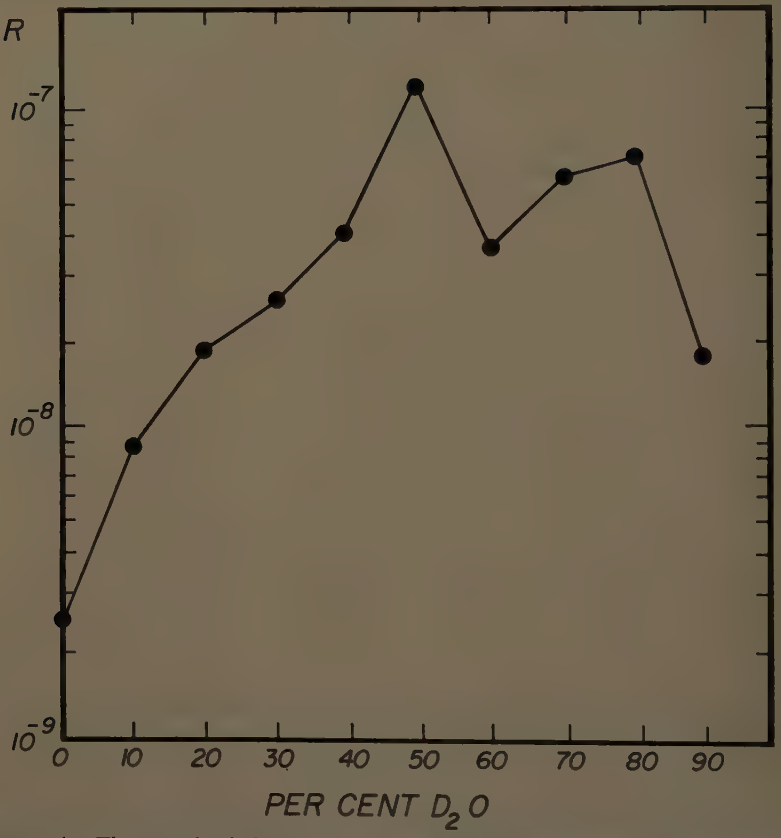

FIGURE 1. The reversion index R of the mutant S-2-16-3 at various D_2O concentrations. The experimental points indicated are the medians of 5 parallel cultures.

In order to exclude the possibility that the increase in the proportion of r^+ revertants found in the stocks of *rII* phage grown in a media containing D_2O is due to a selective advantage enjoyed by the revertants under the altered physiological conditions produced by D_2O, two types of control experiments were performed. In the first, approximately one fifth of the inoculum of both the 50 per cent and the 0 per cent D_2O media were r^+ phage. The ratio of r^+/rII phage was measured at the beginning and end of phage growth in both media. The result of this experiment was that when the initial ratio of r^+/rII was 0.16 and 0.21 in the 50 per cent and 0 per cent D_2O respectively, the corresponding final ratios were 0.15 and 0.22 respectively. Thus the ratio of r^+/rII phage remained constant throughout phage growth in both media.

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* The reversion index is the fraction of r^+ revertants present in a phage stock which has grown from an original inoculum of 10^3 phage per ml. to a final titer of about 10^{10} per ml. (Benzer, 1955).

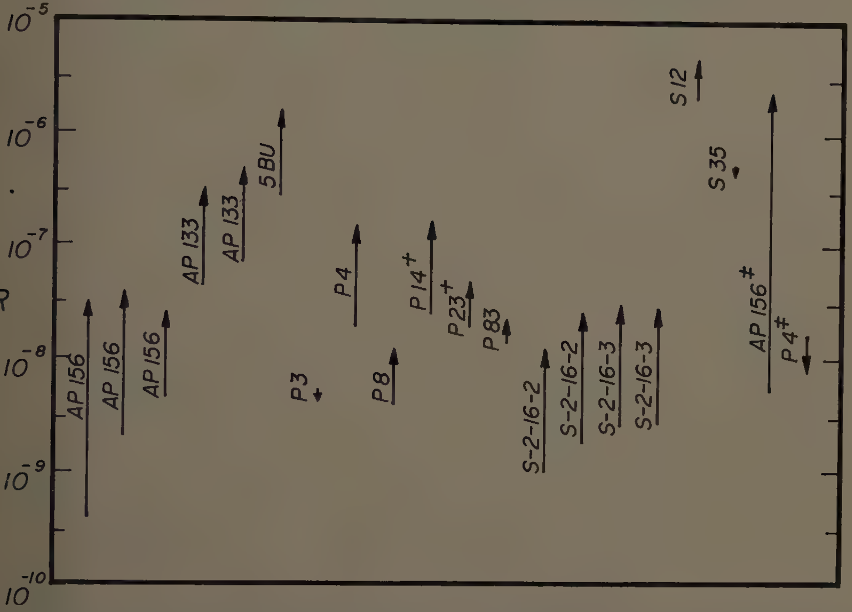

FIGURE 2. The reversion index R is the ratio of r^+/r phage in a 2-ml. culture when the phage inoculum was approximately 1000 r phage. The tail of the arrow represents the reversion index in medium containing 0 per cent D_2O, the head of the arrow the reversion index in medium containing 50 per cent D_2O. The value given is the median of five parallel cultures.

AP 156 and AP 133 are amino purine-induced *rII* mutants supplied by E. Freese, and 5 BU is a 5-bromodeoxyuridine-induced *rII* mutant supplied by D. Pratt. P 3 through P 83 are proflavin-induced *rII* mutants supplied by S. Brenner. S-2-16-2 and S-2-16-3 are *rII* mutants isolated from cultures of r^+ phage grown in media containing 50 per cent D_2O. Because of the small effect of D_2O on the mutation rate it is tentatively assumed that these mutants are of the spontaneous class. S 12 and S 35 are spontaneous *rII* mutants supplied by G. Fermi.

† The values of R given represent the median of 7 parallel cultures.

‡ The head of the arrow represents the reversion index when grown in the presence of 5-bromodeoxyuridine.

The second control experiment is based on the fact that the growth characteristics of the phage may depend on the bacterial host strain. As already stated, the mutant phage *rII* exhibits the *r* plaque type on the indicator strain *E. coli* BS, but gives an r^+ plaque morphology on the related strain BB (McFall and Stent, 1958). If the increase in r^+ revertants in cultures grown in media containing D_2O is due to their selective advantage over the *rII* mutants, then this selection might be less important using a host strain such as BB, on which both r^+ and *rII* phages produce the lysis inhibition phenomenon (Doermann, 1948). To test this possibility, the reversion index of the mutant S-2-16-3 was measured also using BB as the host. The result of this experiment was that the reversion index was 10 times as large in media containing 50 per cent D_2O as in media containing 0 per cent D_2O, which is the same result as that obtained by using strain BS as the host.

FIGURE 2 shows the reversion index for various *rII* mutants grown in media containing 0 per cent and 50 per cent D_2O. It is evident that the reversion index of several mutants, AP 156, AP 133, 5BU, P 4, P 14, S-2-16-2, and S-2-16-3 is increased by about a factor of 10, while the reversion index of mutants P 3, P 8, P 23, P 83, S 12, S 35 appear to be unaffected by 50 per cent D_2O. For some of these mutants the results of several independent determinations of the reversion indices are given, to indicate the reproducibility of these measurements.

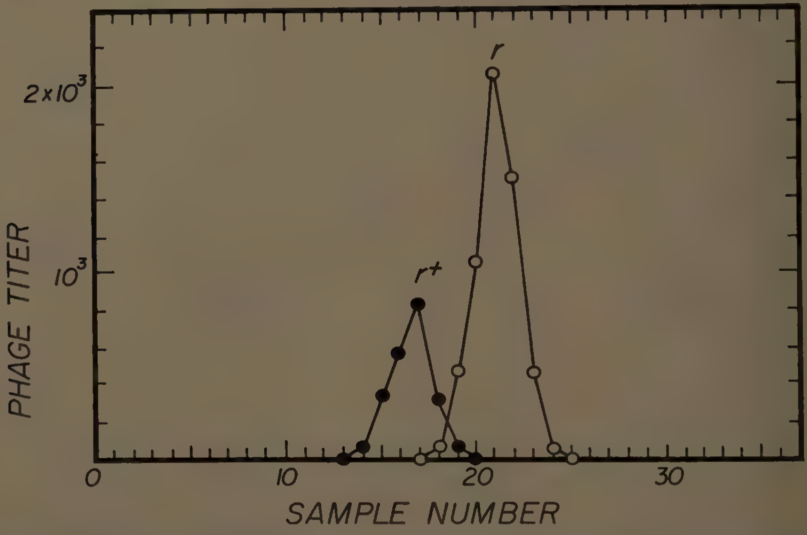

FIGURE 3. The distribution in a CsCl density gradient, created by centrifugation at 30,000 rpm for 16 hours, of r^+ phage grown in medium containing 50 per cent D_2O, and of *r* phage grown in medium containing 0 per cent D_2O.

A solution of CsCl in nutrient broth containing 10^6 phage per ml., is made up to a density of 1.505 gm./ml. and held at 45° C. for one hour. Three ml. of the solution, filling the centrifuge tube to a depth of 3 cm. and covered with mineral oil, are centrifuged in a swinging bucket rotor at 30,000 rpm for 16 hours at 4° C. A small hole is made in the bottom of the centrifuge tube with a pin and the drops are allowed to fall into tubes containing 3 *N* KCl in nutrient broth (Weigle *et al.*, 1959). The solution in each tube is diluted in three equal steps to 0.6 *N* KCl by the addition of nutrient broth. The phage titer in each tube is then assayed on indicator strain BS.

Density of D_2O-grown phage. In order to determine the extent of substitution of deuterium for nonexchangeable hydrogen atoms in the phage, the density of a T4 phage stock grown in 50 per cent D_2O was determined by means of the CsCl density gradient equilibrium sedimentation method (Meselson *et al.*, 1957; Weigle *et al.*, 1959). For this purpose, a mixture of 0 per cent D_2O-

grown T4r and 50 per cent D_2O-grown $T4r^+$ suspended in 4 *M* CsCl were centrifuged until sedimentation equilibrium had been attained and the position of phages in the liquid column determined by dropwise removal of the CsCl solution from the centrifuge tube and assay of the phage content of these drops. The result of this experiment is presented in FIGURE 3, where it may be seen that the two phage populations are separated in the tube by a mean distance that corresponds to a density difference of 0.013 gm./ml.

Assuming that the phage is composed half of protein and half of deoxyribose nucleic acid and using the published compositions of T4 phage protein (Polson and Wyckoff, 1948) and nucleic acid (Wyatt and Cohen, 1953) one may estimate that a phage whose hydrogen atoms are completely replaced by deuterium should be 0.083 gm./ml. more dense than a nondeuterated virus. Some of the deuterium atoms of a D_2O-grown phage, however, are exchangeable with the hydrogen atoms of the aqueous medium in which the particles are suspended during density gradient centrifugation. If one assumes that only those hydrogen atoms bound directly to carbon atoms are not exchangeable in the aqueous medium, then a fully deuterated phage would be 0.062 gm./ml. more dense than a nondeuterated virus. On this basis one may estimate that only 21 per cent of the nonexchangeable hydrogen atoms of the phage grown in 50 per cent D_2O have been replaced by deuterium. This figure of 21 per cent represents a maximum, since some of the hydrogens not bound directly to carbon atoms may also be nonexchangeable, if they participate in hydrogen bonding in the macromolecules of the phage (Hvidt and Linderstrøm-Lang, 1954).

Discussion

Even though no mutagenic effect of 50 per cent D_2O on forward mutation could be detected, it appears that the reverse mutation rate of some, though not all, *rII* mutants to the r^+ wild type can be significantly raised. Nevertheless, even the maximally observed increase in reversion rate of the factor 18 with the mutant AP 156 is very much less than the mutagenic effect of 5-bromodeoxyuridine on this mutant, which causes an increase of the reversion index by a factor of 300 under the present conditions of growth.

The fact that the reversion index of different mutants responds differentially to the D_2O treatment probably reflects some difference in the mutagenic event that occurred during the replication of the viral DNA and that gave rise to the particular mutant genone. According to Freese (1959), phage mutants fall into two classes; the first class represents mutants that have arisen by the replacement of one purine by another purine or one pyrimidine by another pyrimidine; the second class represents mutants that have arisen through the replacement of a purine by a pyrimidine, or vice versa. Amino purine and 5-bromodeoxyuridine are believed to induce mutants of the first class, while proflavin and those agents responsible for most spontaneous mutants induce mutants of the second class. Mutants induced by mutagens for the first class can be reverted only by mutagens for that group; likewise mutants induced by mutagens for the second class can only be reverted by mutagens for the second class. The results shown in FIGURE 2 do not allow D_2O to be assigned to either class of mutagen, for D_2O reverts three mutants out of three tested that had been induced by mutagens of the first class and also reverts 4 out of 10 mutants tested that had been induced by mutagens in the second class. It is then possible that D_2O can induce the nucleotide substitutions characteristic of both types of mutagens.

Acknowledgments

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THE EFFECT OF DEUTERIUM OXIDE ON VIRUS-HOST INTERACTION*

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The toxic effect of high concentrations of deuterium oxide on cells grown in tissue culture was observed as early as 1936.[1] Later investigators[2,3] commented on certain aspects of the cytopathology of deuterium oxide-grown cells (cell clumping, cell enlargement, multiple nuclei, nuclear pyknosis, and vacuolation). We had observed similar changes and had also noted that many of the vacuoles contained lipid. The details of some of the changes in the lipid constitution of normal and D_2O-grown cells are presented elsewhere in these pages (E. L. Rothstein *et al.*). These observations were made early in the course of a general study of the influence of D_2O on the cellular chemistry of various tissue culture cells and the possible effects of these changes on virus reproduction in susceptible cell lines.

Preliminary experiments with the *Escherichia coli* bacteriophage system[4,5] indicated that the influence of deuterium oxide on cell growth and size may indeed affect virus replication. An enhancement of poliovirus replication in D_2O-grown HeLa cells was also noted in the course of our preliminary work.[4]

We shall present here a résumé of our studies of the bacterial system, some aspects of the effects of D_2O on the growth of some mammalian cells cultured *in vitro*, and data that will show the enhancing effects of D_2O on the replication of both virulent and attenuated poliomyelitis virus.

Escherichia coli: T5 and T7 Bacteriophage

E. coli, strain B, was grown aerobically in double-strength nutrient broth in media that had varying concentrations of D_2O. TABLE 1 shows the effect of D_2O on the mass doubling time of the organism and on the lag observed in the growth from a standard inoculum. It may be seen that the mass doubling time increased with increasing D_2O concentration of the growth medium. The final yield of cells at maturity is only slightly decreased, even in 50 per cent of D_2O, although the mass doubling time has doubled.

Normal and partially deuterated *E. coli* were infected with bacteriophage T5 or T7 and viral replication was allowed to continue in either water or deuterated media (TABLE 2). When D_2O was present only during the latent period, the burst size with T5 was decreased, whereas no significant effect was seen with T7. Infected cells grown in heavy water and incubated in normal media show a decreased burst size of questionable significance with T5, and a marked increase with T7. When infected cells were both grown and incubated in deuterated media, T5 multiplication was normal, whereas the burst size seen with

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T7 was significantly increased. With each bacteriophage the latent period was increased: in the case of T5 from 40 to 55 min. and, in that of T7, from 11 to 20 min.

It is known that the medium contributes heavily to the synthesis of T5 DNA,[6] while such is not the case with T7.[7] The reduced burst size observed with T5 may well reflect an inability of the medium to provide precursors of DNA synthesis at an adequate rate. *E. coli* cells grown in deuterated media appear larger than do water-grown cells; after 18 hours many of the cells grown in heavy water media are long and filamentous. It is possible that the increased burst size seen with T7 is due to the increased cell size seen with cells grown in deuterated media.

TABLE 1

EFFECT OF D_2O ON THE GROWTH OF *E. COLI*, B.

D_2O in medium (%)	Time (min.)		Final population (per cent of control)
	Lag	Mass doubling	
0	30	30	100
10	60	33	100
25	60	40	96
50	60	60	92

TABLE 2

EFFECT OF D_2O ON BURST SIZE OF T5 AND T7 BACTERIOPHAGE

D_2O in growth medium (%)	D_2O in medium during latent period (%)	Burst size*	
		T5	T7
0	0	100	100
0	50	50 (P = 0.1)	124 (P = 0.9)
50	0	76 (P = 0.2)	199 (P = 0.1)
50	50	102 (P = 0.9)	134 (P = 0.02)

* Expressed as per cent of H_2O-grown control (T5 = 226; T7 = 209).

Growth of Mammalian Cells in Vitro

The effects of D_2O on the growth of three distinct cell lines have been investigated. The three cell lines have diverse origins: HeLa cells are a stable epithelial line of human origin; L cells are a murine fibroblast strain; L-5178Y is a stable cell line derived from a spontaneously occurring murine leukemia of thymic origin. HeLa and L cells are usually grown as a monolayer on the surface of glass containers; they generally exhibit mass doubling times of 24 to 48 hours. L-5178Y is a round cell that does not stick to glass and therefore can be grown in suspension as either a stationary or agitated culture.[8] It is unique in that it shows an 11.5-hour generation time at 36° C. during logarithmic growth.

The studies with HeLa and L cells were carried out in Leighton tubes. About 150,000 cells were planted per tube in Eagle's basal medium, 10 per cent in calf serum, containing varying amounts of heavy water. The medium in each tube was changed on alternate days. After 8 to 10 days, the cells were harvested and cell yields determined (TABLE 3). It may be seen that 10 per cent heavy water has little effect on the growth rate, whereas 40 and 50 per cent heavy water is quite toxic. As a matter of fact, the final number of cells recovered at these D_2O concentrations was less than the original inoculum.

TABLE 3

EFFECT OF D_2O ON YIELD OF HELA AND L CELLS

Per cent D_2O in growth medium	Total yield of cells*	
	HeLa	L
0	100	100
10	87	85
20	64	54
30	49	34
40	23	16
50	13	4

* As per cent of control.

TABLE 4

EFFECT OF D_2O ON MASS DOUBLING TIME, WET WEIGHT AND DRY WEIGHT OF L5178Y CELLS

D_2O in growth medium (%)	Mass doubling time (hours)	Wet wt. (mg./10^6 cells)	Dry wt. (mg./10^6 cells)
0	11.5	0.71	0.12
5	12.3	0.75	0.12
10	13.0	1.20	0.15
20	14.2	1.20	0.18
30	19.0	1.50	0.15
40	29.0	1.50	0.15
50	56.0	—	

The influence of D_2O on the mass doubling time of logarithmically growing L-5178Y is shown in TABLE 4. With this rapidly growing cell, an effect can be seen even at 5 per cent D_2O. At 30 per cent D_2O, the mass doubling time is almost twice the normal value. At all levels of D_2O below 40 per cent the cells grew logarithmically for several weeks. The value found in 50 per cent D_2O can be determined only over the first 96 hours after exposure of the cells to this medium. During this time the cells grew; then the cell counts began to decrease and the culture died.

A more detailed investigation was made of the effect of D_2O on L-5178Y and HeLa cells. In TABLE 4 are shown the wet weight and dry weight of L-5178Y cells raised in media of varying D_2O concentration. There is a distinct increase in the water content of the cells as the D_2O content is increased. There is also a slight increase in the dry weight of the cells.

HeLa cells were exposed to a medium containing 20 per cent heavy water for varying lengths of time, and the wet weight and dry weight of the cells determined (TABLE 5). Again we see an increase in both the dry weight and water content of the cells. The composition of these deuterated cells with respect to proteins, nucleic acids, and polysaccharides is under investigation at the present time. The lipid composition is reported in another paper in this monograph (E. L. Rothstein *et al.*).

TABLE 5
EFFECT OF 20 PER CENT D_2O ON WET AND DRY WEIGHT OF HELA CELLS

Time of exposure (days)	Control (H_2O)*		D_2O*	
	Wet wt.	Dry wt.	Wet wt.	Dry wt.
3	5.7	0.65	5.2	0.70
6	5.5	0.70	6.5	0.80

* Values are mg./10^6 cells.

TABLE 6
EFFECT OF D_2O ON REPLICATION OF CHAT POLIOVIRUS IN MK AND HELA CELLS

Expt. No.	Temp. (°C.)	Cell type	Cell relationship	Percent D_2O	Burst size ratio D_2O control
1	37	MK	Suspension	40	8
2	37	MK	Monolayer	40	10
3	40	MK	Monolayer	40	50
4	37	HeLa	Suspension	40	5
5	40	HeLa	Suspension	25	10

Poliovirus Multiplication

In a preliminary test of the effect of D_2O on the multiplication of poliovirus type 2, strain MEF-1, it was found that HeLa cells raised and infected in 20 per cent D_2O released 10 times as much virus as did normal cells raised in water-containing media.[4]

A more detailed investigation has been carried out on the multiplication of CHAT,[9] an attenuated type 1 poliomyelitis virus, in HeLa-S3 and in primary cultures of monkey kidney cells. D_2O-containing media was added to the cells 3 to 4 days prior to infection with a high multiplicity of CHAT. Viral multiplication was allowed to proceed in media containing the same D_2O concentrations as those used for cell growth (TABLE 6). The yields of virus obtained from deuterated cells, whether in suspension or in monolayers, were consistently higher than those obtained with controls. The latent period was somewhat longer in D_2O, the highest titers being observed at 8 to 11 hours, in contrast to 6 to 8 hours in standard media.

The incorporation of D_2O in the growth media and the overlay of monkey kidney monolayers resulted in an increase in the average plaque size of both virulent (Mahoney) and attenuated (CHAT) polio strains (TABLE 7). The viral plating efficiency of CHAT on the stable monkey kidney line known as MS (11) is 1 to 5 per cent that observed on primary monkey kidney cells (MK). With D_2O in the growth media and in the agar overlay, the plaque efficiency was increased to between 50 and 100 per cent (TABLE 8).

CHAT virus does not form plaques at 40° C. with doses up to 50,000 PFU,

TABLE 7

EFFECT OF D_2O ON AVERAGE PLAQUE SIZE OF POLIOVIRUS GROWN ON MONKEY KIDNEY

Virus	Per cent D_2O	Plaques measured	Av. size
CHAT	—	120	2.73
CHAT	25	47	4.04
CHAT	40	36	3.96
Mahoney	—	74	3.03
Mahoney	40	51	4.17

TABLE 8

EFFECT OF D_2O (40 TO 50%) ON PLATING EFFICIENCY OF CHAT VIRUS IN MS CELLS

Expt. No.	Cell	Medium	No. plaques
1	MK	H_2O	14
	MS	H_2O	0
	MS	D_2O	14
2	MK	H_2O	33
	MS	H_2O	1
	MS	D_2O	20

TABLE 9

EFFECT OF D_2O ON TEMPERATURE DEPENDENCY OF CHAT POLIOVIRUS REPLICATION ON MK CELLS

Expt. No.	Temp. (°C.)	Medium	Per cent D_2O		No. plaques
			Medium	Overlay	
1	37	H_2O	—	—	19
	37	D_2O	40	50	14
	40	H_2O	—	—	0
	40	D_2O	40	50	12
	40	D_2O	—	50	2
2	37	H_2O	—	—	32, 48
	37	D_2O	25	25	30, 30, 33
	40	H_2O	—	—	0, 0
	40	D_2O	25	25	5, 2, 3
3	37	H_2O	—	—	29, 24, 14
	37	D_2O	25	50	21, 19
	40	H_2O	—	—	0, 0
	40	D_2O	25	50	8, 11, 14
	40	D_2O	—	25	6, 2, 4

a plating efficiency of less than 2 × 10^{-5}. Application of D_2O to the growth media and the overlay was found to cause CHAT plaques to appear at 40° C., with an efficiency 10 to 63 per cent of that observed at 37° C. These data are summarized in TABLE 9.

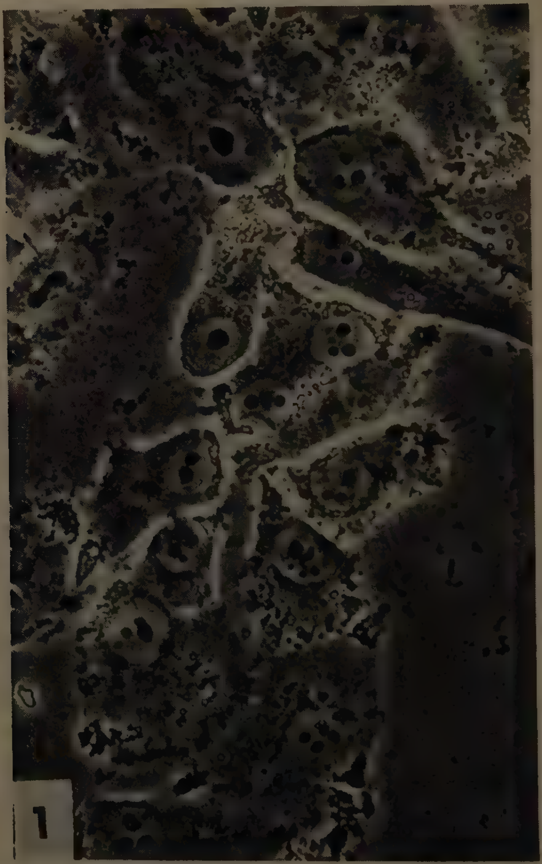

FIGURE 1. HeLa cells in H_2O-containing medium. Cytoplasmic and nuclear characteristics are evident. Phase contrast ×480.

Cytological Observations

The morphological and cytochemical changes induced by D_2O in two cellular systems *in vitro*, HeLa and L cells, are essentially similar. Briefly, in both cell types one can observe a progressive increase in the number of large polynucleated cells paralleling the increase in the concentration of D_2O in the medium. This effect is more evident with the HeLa line, in which multinucleated cells are present in only 1 to 2 per cent of the normal cell population; thus even in 10 per cent D_2O the number of multinucleated cells is increased and, at 40 per cent D_2O, between 30 to 35 per cent of the cell population is made up of large cells with 2 to 3 nuclei, and occasionally more, with an enormous cytoplasm (FIGURES 1 to 4).

The cytoplasm appears as an amorphous protoplasmic mass and, even with phase contrast, very few mitochondria can be discerned; the Golgi areas seem to be hypertrophic. The nuclei are slightly enlarged; no particular variations in the size and number of the nucleoli have been observed so far. The general cytological characteristics of the deuterated cells are, then, very similar to those of the giant cells produced by irradiation. In the case of L cells, enlarged and polynucleated forms are also present, but not to the striking degree observed with HeLa cells; in addition, L cells in 20 per cent D_2O, lose their characteristic cytoplasmic filament, the cell border becoming poorly defined. No significant changes in regard to DNA and RNA were demonstrable by Feulgen

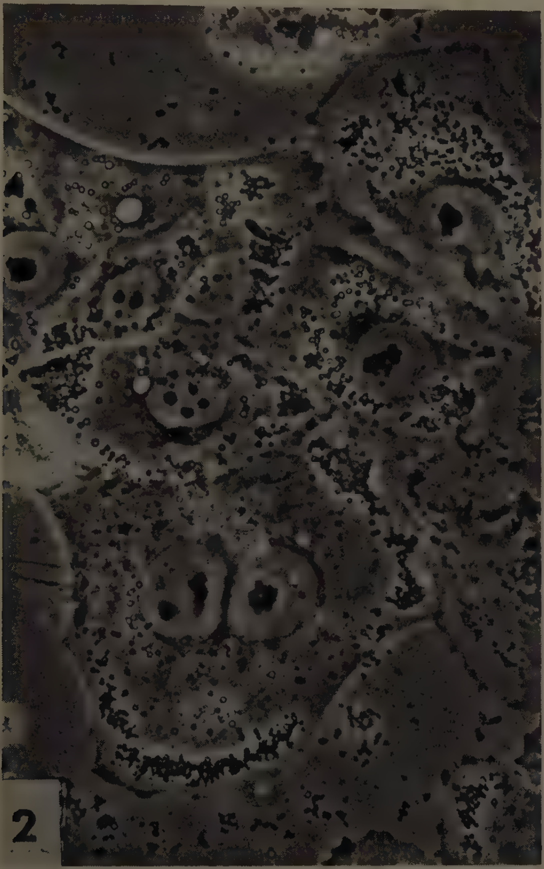

FIGURE 2. HeLa cells in 10 per cent D_2O-containing medium. This field is occupied by a large binucleated cell and by mononucleated cells that show an increased size. In addition, conglomerates of refractile granules (lipochondria) are visible at the periphery of the cytoplasm. Phase contrast ×480.

and pyronin staining. In only one experiment with L cells was there a striking loss of cytoplasmic RNA, unaccompanied by changes in the nucleolar RNA. Lipid stains (Sudan black and Nile blue sulphate) have given equivocal results; in general, we observed a moderate increase of sudanophilic material and a shift in the morphology of this material in the sense that the small, uniform granules—lipochondria—which are present in the normal cells, decrease, while large, irregular masses of sudanophilic material increase. This increase has not been uniform in various experiments and seems to be more evident with L cells. In one HeLa experiment, there was a suggestion of an increase in neutral fat, as demonstrated by Nile blue sulphate. It is probable that the extent of lipid changes is related to the general condition of the cells at the time of deuteration, and possibly to the length of time they are exposed to heavy water.

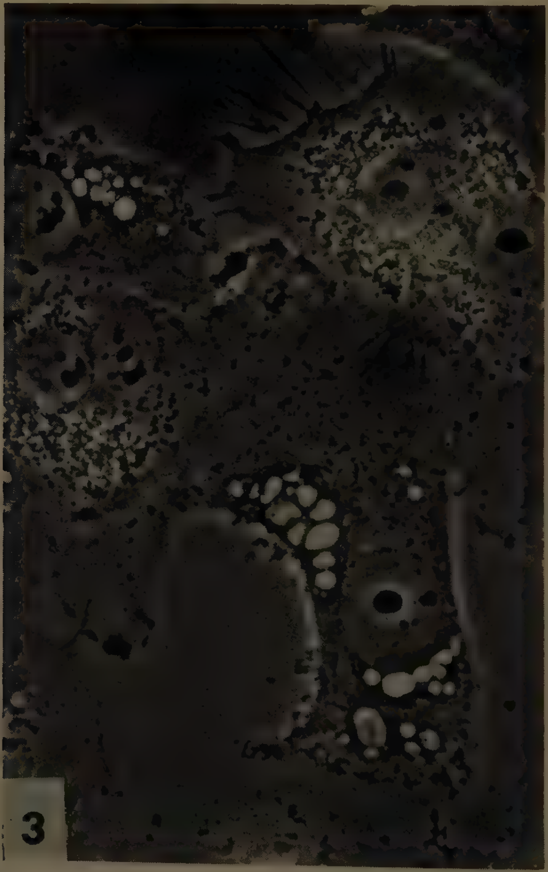

FIGURE 3. HeLa cells in 20 per cent D_2O-containing medium for 7 days. All cells are enlarged and several are binucleated. In the lower portion, a cell with enlarged nucleus and large cytoplasmic vacuoli; these vacuoli are sudanophilic in stained preparations. Phase contrast ×480.

Summary

The effect of D_2O on the growth of *E. coli* and a number of mammalian stable cell lines has been investigated. As the D_2O concentration was increased, the cells of all the species tested appeared larger, showed an increase in water content and dry weight, and a slower growth rate. Cytological investigations showed an increase in the number of multinucleated cells, and a moderate increase in sudanophilic material. In general, cells grown in D_2O exhibited cytological characteristics similar to those observed in giant cells produced by irradiation.[11,12]

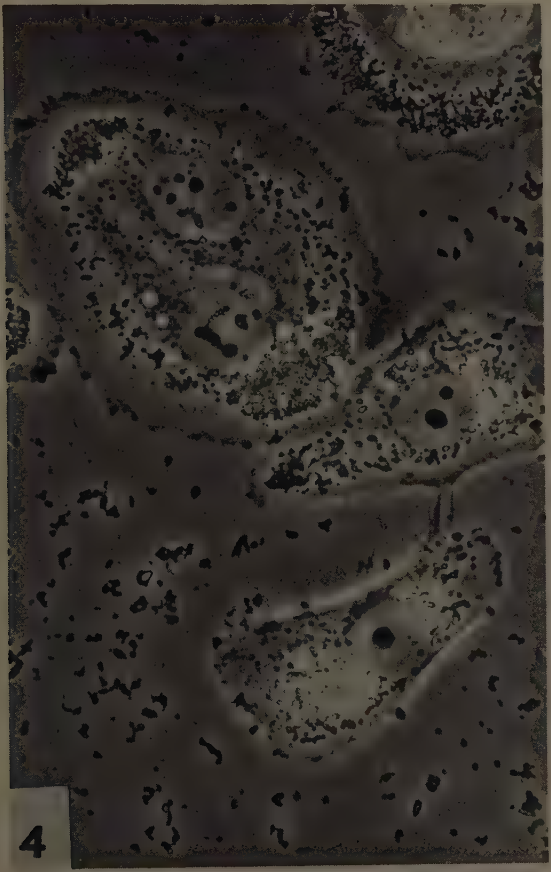

FIGURE 4. HeLa cells in 40 per cent D_2O-containing medium for 7 days. There has been a decrease of cell number, and all the residual cells show a higher degree of the changes illustrated in the previous pictures. Phase contrast ×480.

The effect of D_2O on bacteriophage replication was examined. The burst size of T5 was somewhat reduced in deuterated *E. coli*, but the burst size of T7 was significantly increased. These differences might be explained by the fact that while the bulk of T5 DNA is derived from the medium, most of the T7

DNA is derived from the host. With increased size of the host, one might expect more DNA and protein production. Whether or not deuteration causes an inhibition of the uptake of various substrates from the medium (which could explain the reduced burst size found with T5) is under investigation. Studies of the multiplication of poliovirus in deuterated HeLa and monkey kidney cells also showed a marked increase in burst size. A possible variant of CHAT (attenuated type 1 poliovirus) has been isolated from virus progeny grown in deuterated monkey kidney cells.

The reason for the increased burst size in deuterated cells is not clear at the present time. Similar increases in burst size have been observed in studies of viral replication in X-ray treated giant cells.[13] It is possible that the enlargement of cells grown in D_2O is the underlying factor in the observed increase of poliovirus burst size.

Acknowledgment

We thank G. A. Fischer for his generous gift of L-5178Y and the help he gave us in establishing this cell line in our laboratory.

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STEREOCHEMICAL RELATIONSHIPS IN THE TRICARBOXYLIC ACID CYCLE: MECHANISM OF REACTIONS INVOLVING REVERSIBLE FORMATION OF CARBON-CARBON UNSATURATIONS*

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As a result of unexpected isotope distribution data obtained in studies designed to trace the pathways of intermediary metabolism, it has been realized that in addition to the often observed absolute selectivity of an enzyme for a particular substrate, some enzymes are able to distinguish between chemically-identical groups in apparently symmetrical molecules. As an example of the latter type of specificity one may recall the classical observations on the enzymes concerned in the formation and breakdown of citrate.[1-6] Thus the aconitase-catalyzed elimination of the elements of water from biologically synthesized citrate specifically involves the carboxymethyl group derived from oxaloacetate. The exclusive formation of L-α-glycerophosphate resulting from the phosphorylation of glycerol by adenosine triphosphate in the presence of highly purified preparations of glycerokinase,[7] is indicative of a discrimination between the two hydroxymethyl groups attached to the β-carbon atom of glycerol. Such an asymmetric phosphorylation of the symmetrical glycerol molecule has subsequently been confirmed by the use of isotopes.[8] This type of specificity, usually referred to as stereospecificity, is fully compatible with the geometrical implications of the tetrahedral carbon atom model, and criteria for the "asymmetry" of a carbon atom bearing two like groups have been discussed by Wilcox,[9] by Schwartz and Carter,[10] and most recently by Hirschmann.[11] The original Ogston test of three-point attachment[12] may also be applied, but it is in no way a unique criterion for stereochemical asymmetry.[9-11,13]

In recent years, as a result of the increased application of the hydrogen isotope technique in studies on isolated enzyme systems, a similar enzymatic nonequivalence has been established for carbon-bound hydrogen atoms. Studies on various enzymatically catalyzed oxidation-reduction reactions involving hydrogen transfers to and from pyridine nucleotides, in addition to firmly establishing the concept of direct hydrogen transfer, have also demonstrated that these reactions are stereospecific with respect to the pyridine nucleotide.[14-17] The reaction catalyzed by alcohol dehydrogenase was also shown to behave stereospecifically with respect to ethanol.[18] A pure enantiomorph of 1-deuteroethanol has been obtained by the enzymatic reduction of 1-deuteroacetaldehyde in the presence of reduced diphosphopyridine nucleotide (DPNH) and, from the measured optical rotation, its absolute configuration has been deduced.[19] Phosphoglucose isomerase and phosphomannose isomerase have been shown to be able to distinguish between the 2 hydrogen atoms on the C-1 of fructose-6-phosphate; in addition, the hydrogen atom that is acted upon by one isomerase is not the one that is activated by the other isomerase.[20] In an analogous reaction, the enzyme catalyzing the reversible isomerization of ribulose-5-phosphate to ribose-5-phosphate has been reported to act stereospecifically as regards the hydrogen atoms on C_1 of ribulose-5-phosphate, as evidenced by the incorporation of only one atom of tritium per molecule of pentose* when the interconversion is carried out in tritiated water.[21] Muscle aldolase and triosephosphate isomerase have been found to catalyze a stereospecific exchange of one of the hydrogens bound to the carbinol carbon of dihydroxyacetone phosphate[22-24] and, furthermore, the exchange catalyzed by the two enzymes was found to involve different hydrogen atoms.[23-25] Recently Rose[26] also established the absolute configuration of the two enantiomorphs of monotritiodihydroxyacetone phosphate obtained by the aldolase- and triosephosphate isomerase-catalyzed exchange reactions in tritium oxide. Equilibration of β-ditritio-α-ketoglutarate with isocitric acid dehydrogenase results in the labilization of only one atom of tritium, and this evidence therefore points to a stereospecific oxidative decarboxylation of *d*-isocitrate as catalyzed by this enzyme.[27] The stereospecificity of amino acid decarboxylations has been ascertained from similar lines of evidence.[28] The aspartase-catalyzed amination of fumarate in D_2O results in the formation of 3-monodeutero-L-aspartate, indicating that the addition of hydrogen to the β-carbon atom is stereospecific.[29,30]

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It is the purpose of this paper to review in detail some observations on the reactions within the tricarboxylic acid cycle involving the reversible formation of carbon-carbon unsaturations. The results of a study on an anaerobic exchange of deuterium from the medium into succinate, as catalyzed by heart particle succinic oxidase preparations,[31,32] will be discussed in terms of the stereochemistry of the succinic dehydrogenase reaction. The stereospecific nature of the reversible hydrations of fumarate[31-33] and *cis*-aconitate[34] will be described. The theoretical implications of this observation, as regards the mechanism of the enzymatically catalyzed reactions, will be considered. Evidence will be presented that establishes the configuration of the product of the stereospecific enzymatic hydration of fumarate in D_2O in relation to the stereospecificity of the aconitase-catalyzed reaction.[35,36] The consequences of this configurational interrelationship will be shown to provide evidence for a *trans* fumarase-catalyzed addition reaction.[36]

Succinic Dehydrogenase

Mechanism of anaerobic labeling of succinate. Attempts to study with deuterium the possible mechanism of an anaerobic hydrogen transfer reaction from reduced diphosphopyridine nucleotide to fumarate, as catalyzed by heart particle preparations,[37] necessitated an investigation of the possibility that these preparations, which contain succinic dehydrogenase, also catalyzed an anaerobic exchange reaction described by Weinmann *et al.*[38] These workers observed that, when dideuterosuccinate was incubated anaerobically with a rabbit kidney succinic dehydrogenase preparation, there resulted a significant exchange of the hydrogen atoms of the medium with the deuterium atoms of the dideuterosuccinate. They also noted that this rate of exchange, although not influenced by the presence of methylene blue, was roughly equivalent to the rate of succinate oxidation in the presence of a suitable acceptor such as methylene blue (MB). This correspondence between the rates of anaerobic exchange and oxidation was interpreted as lending support to the hypothesis that the same enzyme, namely succinic dehydrogenase, catalyzed both of the following reactions: (1) oxidation of succinate and (2) hydrogen exchange.

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* In this paper consideration is given only to tritium or deuterium atoms stably bound to carbon, since an isotope attached to oxygen or nitrogen readily exchanges with water and is lost in the course of the isolation procedures.

TABLE 1

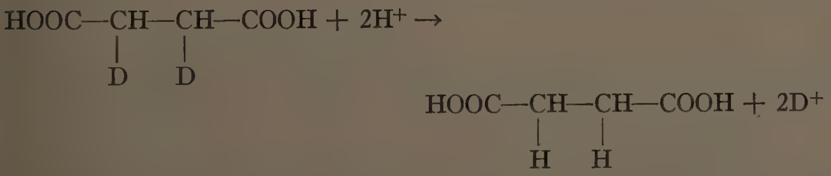
ANAEROBIC DEUTERIUM INCORPORATION INTO SUCCINATE, FUMARATE, AND MALATE BY KEILIN-HARTREE HEART-PARTICLE PREPARATION*

Expt.	Acids added initially	Acids isolated at end of reaction	Atom deuterium per molecule
1	1 mmole succinic	Succinic	0.031
2	1 mmole succinic + 1 mmole fumaric	Succinic	0.460
		Fumaric	0.019
		Malic	0.779

The reaction mixture contained 2.44 mmoles of potassium phosphate buffer, *p*H 7.29; 11.0 mg. of cytochrome c; 1.0 ml. of an enzyme preparation capable of oxidizing, under similar conditions, 418 μmoles of succinic acid per hour with succinate as the sole substrate, or 319 μmoles of succinic acid per hour when succinate plus fumarate were present; the fumarase equilibrium was established about the fourth hour. The total volume was 16.5 ml., having a D_2O concentration by volume of 88.2 and 86.3 per cent for experiments 1 and 2, respectively. Succinate was tipped into the main body of the Thunberg tube after the reaction mixture had been flushed with purified nitrogen gas and the tubes had been evacuated with an oil pump. Incubation time, 8 hours; temperature, 37° C.

* Data from Englard and Colowick.[31,32]

$$\mathrm{HOOC{-}CH_2{-}CH_2{-}COOH + MB \rightarrow HOOC{-}CH{=}CH{-}COOH + MBH_2} \quad (1)$$



$$\mathrm{HOOC{-}\underset{D}{\underset{|}{CH}}{-}\underset{D}{\underset{|}{CH}}{-}COOH + 2H^+ \rightarrow HOOC{-}\underset{H}{\underset{|}{CH}}{-}\underset{H}{\underset{|}{CH}}{-}COOH + 2D^+} \quad (2)$$

When normal succinate was incubated anaerobically in a medium containing D_2O and a Keilin-Hartree heart-particle preparation, made as described by Slater,[39] the exchange reaction was extremely slow compared with the possible rate of aerobic succinate oxidation (TABLE 1, Experiment 1). However, it can be seen that initially-added fumarate greatly stimulated the incorporation of deuterium into succinate (TABLE 1, Experiment 2). Since other observations indicated that the deuterium appearing in the succinate arose directly from the medium and not from a reduction of labeled fumarate, the results are most readily explained by the following equations:

$$\mathrm{^-OOC{-}CH_2{-}CH_2{-}COO^- + 2X \rightarrow {}^-OOC{-}CH{=}CH{-}COO^- + 2X^- + 2H^+} \quad (3)$$

$$\mathrm{^-OOC{-}CH{=}CH{-}COO^- + 2X^- + 2D^+ \rightarrow {}^-OOC{-}CHD{-}CHD{-}COO^- + 2X} \quad (4)$$

EQUATION **3** represents a reduction of a natural carrier X by succinate, and EQUATION **4** represents the reversal of this reaction in heavy water. Alternatively, the reduction of X would yield XH_2, the hydrogens of which would be rapidly exchangeable with the medium D_2O. The resulting XD_2 would subsequently be reoxidized by fumarate, yielding X and deuterosuccinate. Thus the exchange reaction studied here appeared to be not a result of an increased rate of dissociation of the C—H bonds of succinate, as suggested in EQUATION **2**, but rather a reflection of the oxidation of succinate by a natural carrier in such a manner that the hydrogens of succinate are directly or indirectly donated to the medium. This possibility was also considered by Weinmann *et al.*,[38] and earlier by Erlenmeyer *et al.*[40] According to this scheme, the addition of fumarate would promote the incorporation of deuterium into succinate by establishing a new equilibrium position in which the rates of both REACTIONS **3** and **4** would be increased.* Since relatively crude preparations of enzyme were used in this study, the available data did not permit a definitive conclusion as to the involvement of only one enzyme in the catalysis of both REACTIONS **3** and **4**. Thus while REACTION **3** in all probability is due to the generally described succinic dehydrogenase, the possibility must be entertained that REACTION **4** may be catalyzed by an enzyme similar to the fumaric reductase isolated from yeast by Fischer *et al.*[41-43] However, in such a case, the carrier X would have to be capable of interacting with both enzyme systems. The finding that a highly purified flavoprotein from beef heart shows both succinic dehydrogenase and fumaric reductase activity, and that the reduced enzyme can be reoxidized by fumarate,[44] supports the view that a single enzyme is responsible for EQUATIONS **3** and **4**.

TABLE 2

ENZYMATIC OXIDATION OF ENZYMATICALLY LABELED DEUTEROSUCCINATE*

Acids	Atom deuterium per molecule
Deuterosuccinate obtained from an anaerobic enzymatic exchange reaction	0.100
Succinate after 50 per cent of above deuterosuccinate was enzymatically oxidized	0.135
Fumarate resulting from the deuterosuccinate oxidation	0.044

* Data from Englard and Colowick.[31,32]

Deuterium distribution in enzymatically produced deuterosuccinate. On the assumption that the anaerobic exchange reaction is catalyzed by a single enzyme and that the same enzyme is concerned in the aerobic oxidation of succinate, the occurrence of a sterically specific behavior for succinic dehydrogenase could be tested. Thus if the exchange reaction resulted in succinate labeled specifically in two of its four methylene hydrogen positions, its oxidation by the very same enzyme should yield essentially unlabeled fumarate. TABLE 2 records the results of such an experiment, in which deuterosuccinate, obtained by enzymatic exchange of deuterium into normal succinate under conditions similar to those described in TABLE 1, Experiment 2, was enzymatically oxidized to fumarate. The results clearly indicate that the resulting fumarate retained (per molecule) about half the deuterium originally present in the succinate. These findings, therefore, lend themselves to the interpretation that during the course of the anaerobic exchange reaction all four methylene hydrogens of succinate became labeled. The apparent discrepancy between the deuterium content of succinate before and after 50 per cent oxidation is probably due to an isotope effect that causes preferential oxidation of the unlabeled succinate molecules[45] leading to a higher apparent deuterium content of the residual succinate and a somewhat lower than theoretical content of the resulting fumarate.

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* At the new equilibrium position, REACTION **4** would be increased in rate simply because of the increased fumarate concentration, while REACTION **3** would be increased in rate because of the increased concentration of oxidized carrier resulting from fumarate addition.

Steric course of succinic dehydrogenase. In order to aid in the discussion of the stereochemistry of this process, attention is called to the succinic acid model depicted in FIGURE 1. In this diagram, the hydrogen atoms marked H_1 and H'_1 are stereochemically indistinguishable. The atoms marked H_2 and H'_2 are also stereochemically indistinguishable. H_1 or H'_1 is stereochemically readily distinguishable from H_2 or H'_2 by the following test: when examined from a point outside the molecule on the axis joining the methylene carbon atoms, the H_1, H_2, and COOH groups attached to the proximal methylene carbon will always appear in that order, reading clockwise.

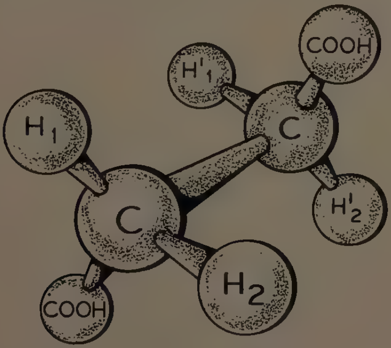

FIGURE 1. Succinic acid model.

Whenever dehydrogenation occurs by removal of a pair $H_1H'_2$, this is stereochemically indistinguishable from removal of the pair H'_1H_2, and there is no enzyme or other device that could make that distinction. On the other hand, when dehydrogenation occurs by removal of the pair $H_1H'_1$, this is stereochemically readily distinguishable from removal of the pair $H_2H'_2$, and an enzyme could readily make that distinction. The fact that the enzyme studied here does not distinguish between the 4 hydrogen atoms of succinic acid must mean either that (1) the enzyme is catalyzing the removal of a pair $H_1H'_2$ ($= H'_1H_2$), in which case there is no chance for selective action on one pair of hydrogens, or that (2) the enzyme is catalyzing indiscriminately the removal of either an $H_1H'_1$ or an $H_2H'_2$ pair, in spite of the fact that there is a theoretical possibility for selective action.

Thus far no statement has been made regarding the configuration of the carboxyl groups of the succinate or the steric mechanism of the reaction, since the argument is independent of these considerations. If the staggered configuration is assumed, (1) corresponds to a *trans* elimination of two hydrogen atoms, $H_1H'_2$ (= H'_1H_2), whereas (2) would correspond to the indiscriminate *cis* elimination of pairs $H_1H'_1$ and $H_2H'_2$.

The consequences of a *trans* versus *cis* mechanism of fumarate reduction in the back reaction (EQUATION 4) are illustrated in FIGURE 2. The projection formula for fumarate (lying in a plane perpendicular to the plane of the paper) attempts to show the planar structure of the molecule and the *trans* configuration of the carboxyl groups. The dideuterosuccinate isomers are depicted with their α and α' carbon atoms projecting forward and behind from the plane of the paper. The product of the reaction occurring by a stereospecific (or nonstereospecific) *trans* mechanism of addition always yields mesodideuterosuccinate. A racemic mixture of two optically-active forms of dideuterosuccinate, however, is obtained by an unspecific *cis* addition. Only a stereospecific *cis* mechanism of addition would yield either D- or L-dideuterosuccinate, depending on which side of the fumarate molecule the two deuterium atoms are added.

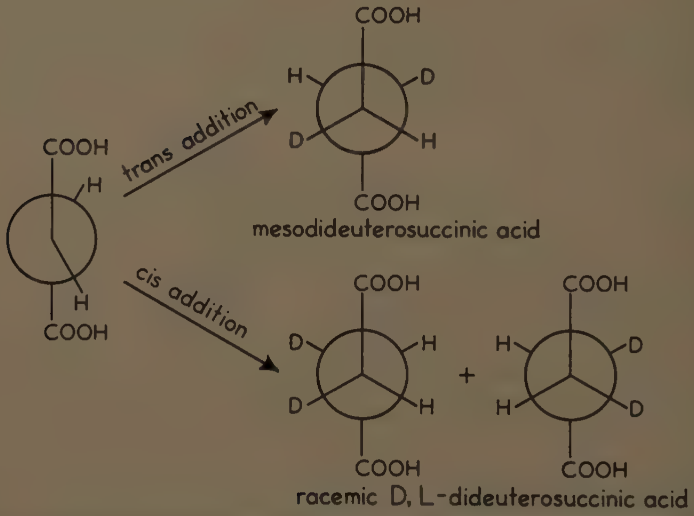

FIGURE 2. Possible isomeric forms of dideuterosuccinic acid resulting from the deuteration of fumaric acid.

In view of the fact that other enzymes exert stereospecificity with respect to methylene hydrogens in most cases thus far studied (with the possible exception of a few[16,46-49]), and since enzymatic reactions as a rule do not yield racemic mixtures, it appears more likely that a *trans* mechanism is operative in the reaction catalyzed by succinic dehydrogenase,[32] whether one considers the elimination reaction from succinate or the addition reaction to fumarate. Conclusive evidence for a *trans* mechanism for succinic dehydrogenase has recently been obtained by H. van Milligan and T. T. Tchenn (personal communication, 1960), thus confirming the tentative conclusions based on the theoretical stereochemical implications of the results obtained in the anaerobic exchange reaction.

Fumarase

Stereospecificity of the reaction. The enzyme preparation used in the experiments just described contained sufficient fumarase to establish the fumarate-malate equilibrium. Hence the finding of deuterium in succinate and the stimulation of this deuterium incorporation by fumarate may have reflected an initial incorporation of deuterium into fumarate via the fumarase equilibrium, provided that this enzyme behaved in a nonstereospecific manner with respect to the hydrogen atoms on the methylene carbon of L-malate. To test this possibility, all three acids from the reaction mixture, in which both succinate and fumarate were incubated anaerobically with the succinic dehydrogenase preparation in D_2O, were isolated, and their respective deuterium content was determined. The results are outlined in TABLE 1, Experiment 2. Thus while the L-malate exhibited a high deuterium content, as was anticipated, the fumarate had an exceedingly low deuterium content.* Since the reaction proceeded long after the fumarase equilibrium had been reached, ample opportunity had been allowed for the fumarate to become more highly labeled if the fumarase had behaved nonstereospecifically. Identical results were obtained from experiments in which fumarate alone was incubated with the enzyme preparation under conditions identical to those in which the exchange reaction was studied. At the same time, Alberty and his associates,[33] pursuing their brilliant and intensive studies on the mechanism of the fumarase reaction, reported similar results with crystalline preparations of the enzyme. These results thus tend to strengthen the belief that the deuterium incorporated anaerobically into succinate in the absence or presence of fumarate arises directly from the medium and not from labeled fumarate.

The finding that fumarase in its back reaction behaves with strict specificity in removing the identical molecule of water from L-malate as was incorporated into fumarate in its forward reaction, is in accordance with the equation in FIGURE 3 showing that the enzymatic equilibration of fumarate in D_2O leads exclusively to 3-monodeutero-L-malate and unlabeled fumarate. The reaction is therefore stereospecific not only for the addition of the OH group in the formation of the asymmetric α-carbon of L-malate, but also for the concomitant addition of the hydrogen atom to the β-carbon atom in the formation of the methylene group.

In addition, FIGURE 3 also shows the two possible isomeric forms of 3-monodeutero-L-malate depending on the mechanism of the enzymatically catalyzed addition reaction to fumarate. The 3-monodeutero-L-malate isomers are depicted with their α- and β-carbon atoms projecting forward and behind, respectively, from the plane of the paper, and differ only with respect to the β-carbon atom configuration, the assignment of the indicated absolute configuration for the α-carbon atoms being dictated by the known specificity of the enzyme for the L-isomer. With the α- and β-carbon carboxyl groups in a *trans* configuration, as indicated, the two isomers are distinguished by the relative proximity of the two carbon bound protons originating from fumarate. A *trans* mechanism of D_2O addition yields a product in which the two protons are *trans* to each other, whereas the same two protons are *gauche* to one another in the isomer arising from a *cis* mechanism of D_2O addition.

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* The small amount of deuterium found in the fumarate in Experiment 2 of TABLE 1 probably arose from the initially-formed deuterosuccinate by a "nonstereospecific" reversal of REACTION 4, and its low value probably reflects the dilution of the isotope by the initially-added normal fumarate. However in other experiments, a greater incorporation of label into fumarate was observed, the reasons for which are not clear.[31,32] It was found, however, that this increased labeling depended on the addition of succinate, as would be expected from the proposed scheme for the mechanism of the anaerobic exchange reaction.

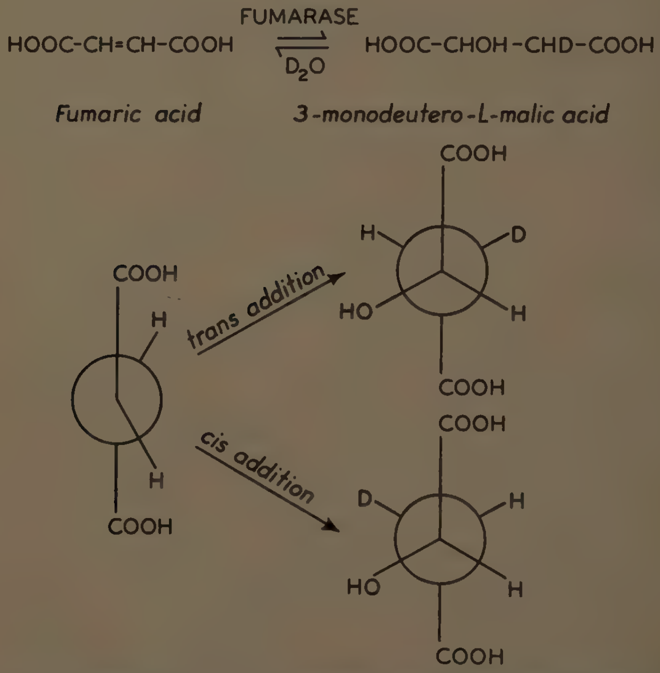

FIGURE 3. Stereospecificity of the fumarase reaction and possible isomeric forms of 3-monodeutero-L-malic acid.

Based on the observed dipolar broadening of the proton magnetic resonance absorption in solid 3-monodeutero-L-malate obtained by the enzymatic hydration of fumarate in D_2O,* Farrar *et al.*[50] concluded that the α- and β-carbon protons are *gauche* to one another. On the assumption that the carboxyl groups are *trans* in solid L-malic acid, the observed data were interpreted as being compatible only with a *cis* mechanism of addition, catalyzed by the enzyme fumarase. The results to be presented in a later section of this paper, however, are at variance with this latter conclusion and the nature of the steric course of the fumarase reaction will therefore be reconsidered in the light of more recent evidence.

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* The hydroxyl and carboxyl protons were exchanged in D_2O to yield DOOC—CH(D)—CH(OD)—COOD, so that the protons in question are the important magnetic nuclei.

Aconitase

Stereospecificity of the reaction. In the course of their studies on the mechanism and stereospecificity of the pyridine nucleotide transhydrogenase reaction, San Pietro *et al.*[51] observed that incubation of diphosphopyridine nucleotide (DPN^+), isocitrate, transhydrogenase, isocitric dehydrogenase, and catalytic amounts of triphosphopyridine nucleotide (TPN^+), in a medium containing D_2O, led to the appearance of deuterated reduced diphosphopyridine nucleotide (DPND), which is formed as follows:

$$\text{Isocitrate} + TPN^+ \rightleftharpoons \alpha\text{-ketoglutarate} + CO_2 + TPNH + H^+ \qquad (5)$$

$$TPNH + DPN^+ \rightleftharpoons TPN^+ + DPNH \qquad (6)$$

Since other results were consistent with the view that REACTION 6, catalyzed by transhydrogenase, occurred by a direct hydrogen transfer between reduced and oxidized nucleotides,[51] the assumption was made that in the above-mentioned experiment deuterated reduced triphosphopyridine nucleotide (TPND) was continuously generated by the isocitric acid dehydrogenase preparations (presumably contaminated with aconitase), which had been used to catalyze REACTION 5. The enzymatic reduction of TPN^+ by unlabeled isocitrate in a medium enriched with D_2O was subsequently found to yield unlabeled reduced triphosphopyridine nucleotide (TPNH),[34] indicating that, like many other dehydrogenases, TPN-isocitric acid dehydrogenase catalyzes a direct transfer of hydrogen from *d*-isocitrate to the oxidized nucleotide. The possibility of a nonstereospecific aconitase reaction that could have accounted for the results obtained by San Pietro *et al.*[51] was therefore examined experimentally.

The results, outlined in TABLE 3, indicate that no deuterium is incorporated from heavy water into reduced TPN during the action of pig heart-isocitric acid dehydrogenase, even under conditions in which the citrate (and presumably the isocitrate) is labeled by the action of aconitase.* Thus while incubation of citrate with aconitase led to a continual introduction of deuterium into citrate, no such increase with time was observed in the deuterium content of the TPNH obtained by the subsequent action of isocitric acid dehydrogenase on the *d*-isocitrate present in the corresponding equilibration mixtures. If the aconitase were not stereospecific, such deuterated citrate molecules should have yielded *d*-isocitrate molecules with deuterium on all positions where a subsequent direct transfer of hydrogen to TPN^+ could occur and, accordingly, such a reaction should have yielded TPND. The results, therefore, definitely establish the stereospecific behavior of aconitase with respect to the methylene hydrogen eliminated in the conversion of citrate to *cis*-aconitate.

Another argument supporting the view that aconitase is stereospecific may be based on a consideration of the quantitative aspects of the extent of labeling of the citrate. The incubations of citrate with aconitase were carried out in a medium containing 85 per cent D_2O. A stereospecific mechanism should lead maximally to the introduction of 0.85 atom of stably-bound deuterium per molecule of citrate, whereas a nonstereospecific reaction should lead maximally to 1.70 atoms of deuterium per molecule of citrate, the fractionation due to isotope effects being neglected. If the value of 0.546 obtained after 8 hours is taken to be close to the equilibrium value, one may conclude that the reaction is occurring stereospecifically and, furthermore, that there is an isotope effect, K_H/K_D, of $85(1 - 0.546)/15 \times 0.546$, or 4.7 in the hydration of aconitase. Since this figure is in the range of K_H/K_D values for other reactions,[52] it is suggested that the making of a C—H bond is rate-limiting in the hydration of aconitate. It is also suggested that the breaking of a C—H bond is not rate-limiting in the dehydration of citrate since, if it were, the above isotope effect should not have been observed.

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* The labeling of the pyridine nucleotide fraction in the experiments of San Pietro *et al.*[51] has been found to be an artifact resulting from the boiling step in the analytical procedure.[34]

TABLE 3
EFFECT OF PRELIMINARY INCUBATION WITH ACONITASE ON DEUTERIUM INCORPORATION INTO CITRATE AND TPNH*

Sample No.	Time of preliminary incubation with aconitase (min.)	Dilution factor for nicotinamide samples	Deuterium content			
			TPN		Citrate	
			Atom per cent excess†	Atom deuterium per molecule‡	Atom per cent excess†	Atom deuterium per molecule‡
1	20	10.6	0.040	0.025	0.211	0.093
2	95	13.7	0.052	0.043	0.781	0.344
3	210	16.4	0.038	0.037	0.969	0.426
4	315	16.7	0.015	0.015	1.190	0.524
5	480	20.0	0.022	0.026	1.240	0.546

The complete system contained 5.95 mmoles of potassium citrate, *p*H 7.44, 2.5 mmoles of glycylglycine buffer, *p*H 7.42, and 5 ml. of aconitase preparation, for each 100 ml. of reaction mixture, at a D_2O concentration of 85 per cent by volume. The reaction mixture was incubated at 30° C.; at various time intervals over the 8-hour period, 0.2 ml. aliquots were removed and added to 2.8 ml. of H_2O, and the absorption was determined at a wave length of 240 mμ in a Beckman spectrophotometer, to measure *cis*-aconitate formation. It was thus determined that the equilibrium was reached within 25 min., as the maximal E_{240} of 0.662 did not change beyond this time. At indicated times, 20 ml. aliquots were removed and immersed in a boiling-water bath for 2 min. with immediate subsequent cooling in an ice bath. These aliquots were filtered and made up to 26.5 ml. with H_2O. Of these diluted aliquots, 20 ml., containing approximately 63 to 71 μmoles of *d*-isocitrate, were added to 250 μmoles of glycylglycine buffer, *p*H 7.42, 16.2 μmoles of $MnCl_2$, 0.50 ml. of isocitric acid dehydrogenase, and approximately 52 μmoles of unneutralized TPN^+; the final volumes were adjusted to 27 ml. The D_2O concentration at this time was 47.6 per cent by volume. The incubations were carried out at 30° C.; complete reduction of the nucleotide was achieved within 10 min. At exactly 11 min., the reactions were stopped by heat inactivation. Nicotinamide from the chemically-reoxidized reduced TPN and the residual citrate were isolated and analyzed for deuterium.

* Data from Englard and Colowick.[34]

† Experimental values.

‡ Values corrected for dilution (when applicable) and calculated on the basis that 1 atom of deuterium per molecule of nicotinamide and quinidine citrate corresponds to values of 16.7 and 2.28 atom per cent excess, respectively.

The mechanism of the aconitase reaction, based on the experiment just described modified in accordance with the scheme proposed by Speyer and Dickman,[53] and illustrating stereospecific behavior, is depicted in FIGURE 4. The demonstrated stereospecificity is seen to consist of a selective action on one of the pair of methylene hydrogens on carbonium ion 1 arising from citrate. As illustrated here, this selectivity is considered to apply not only to the dissociation of the deuterium as a deuteron to reform free unlabeled *cis*-aconitate, but also to the intramolecular transfer of the deuterium to the β-carbon[53] to form carbonium ion 2, the immediate precursor of *d*-isocitrate. Thus the α position of monodeuteroisocitrate arising in D_2O directly or indirectly via free *cis*-aconitate from the aconitase-produced monodeuterocitrate contains only hydrogen. Since the isocitrate must have contained deuterium on the β-carbon, the absence of deuterium in the reduced TPN must mean that it is the hydrogen on the α-carbon that is transferred directly to TPN^+ by the isocitric acid dehydrogenase reaction,* and that this hydrogen originated from the α-H of citrate, which did not participate in the reversible dehydration catalyzed by aconitase.

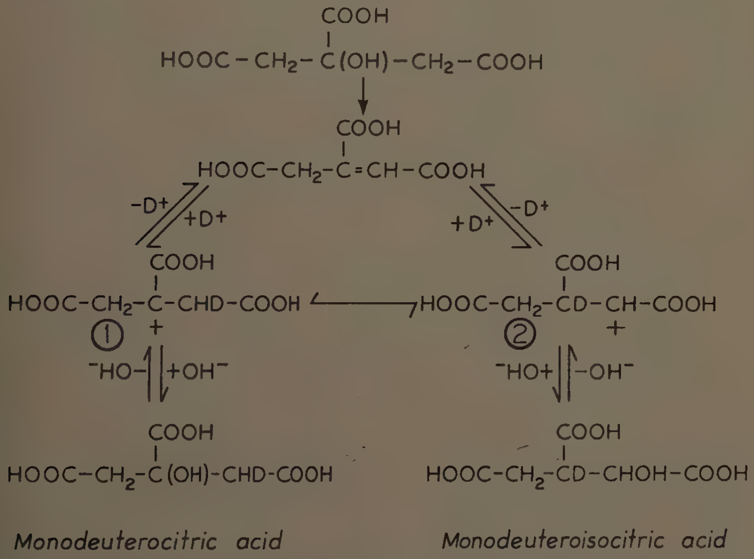

FIGURE 4. Mechanism of aconitase action, illustrating stereospecific behavior (Speyer and Dickman[53]; England and Colowick[34]).

Steric course of the aconitase reaction. Since isocitrate contains two asymmetric carbon atoms in the classical sense, and citrate, when subjected to the action of aconitase, is handled asymmetrically both with respect to the carboxymethyl groups attached to the β-carbon atom[3-6] and the hydrogen atoms of the α methylene carbon,[34] four possible isomers of each acid can exist. These are shown in FIGURE 5 as arising from either a *cis* or *trans* mechanism of hydration of *cis*-aconitase in D_2O, each mode of addition yielding a particular racemate. With the α- and β-carbon carboxyl groups in a staggered configuration, the products of a *trans* mechanism of D_2O addition have the original proton and carboxymethyl group of *cis*-aconitate in a *gauche* configuration, whereas these

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* This conclusion is compatible with the view that the first product of the oxidation is the keto form of oxalosuccinate, but does not rule out the primary formation of the enol form that, from the present data, can arise from *d*-isocitrate only by the direct transfer of the α-carbon-bound hydrogen to TPN^+ and the loss of the β-carbon-bound hydrogen (deuterium) to the medium as a proton.

2 groupings are *trans* to each other in the products arising by a *cis* mechanism of D_2O addition. It should be remembered, however, that for either citrate or isocitrate only one of the represented structures will conform with the known specificity of the aconitase reaction. Fortunately, the absolute configuration of naturally-occurring *d*-isocitrate is known, and one can therefore reach a conclusion as to the steric course of the aconitase catalyzed reaction. This is illustrated in FIGURE 6.

On the basis of physical and synthetic evidence, Gawron and his associates[54,55] have concluded that the β-carboxyl in D,L isocitric acid lactone is *cis* to the α-carboxyl group. Since the stereochemistry of the α-carbon of natural dextrorotatory isocitrate had been previously elucidated by the work of Greenstein *et al.*[56] and Winitz *et al.*[57] and shown to correspond to the L_s configuration, Gawron and his co-workers,[54,55] referring to the β-carboxyl group of isocitrate as a substituent similar to the β-hydroxyl group of threonine, assigned to the β-carbon, by virtue of the above-mentioned evidence, the D_s* configuration.

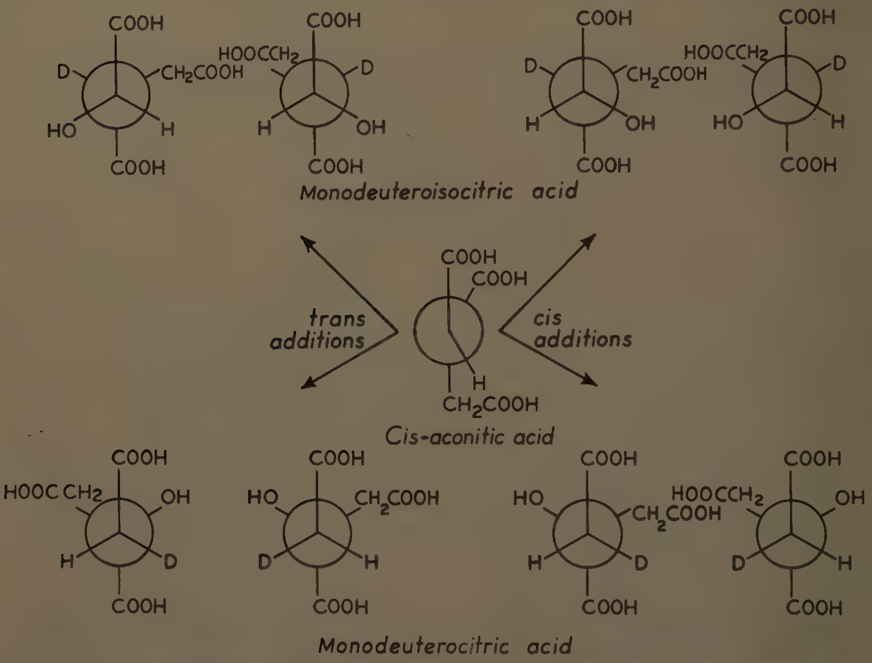

FIGURE 5. Possible isomeric forms of monodeuterocitric and monodeuteroisocitric acids resulting from the hydration of *cis*-aconitic acid in D_2O.

* Thus naturally-occurring *d*-isocitrate has been designated as αL_s-βD_s-isocitrate by Gawron *et al.*,[54,55] and this nomenclature represents a slight modification of that used by Winitz *et al.*[57] For the purpose of a paper discussing the possible configurational interrelationships between the products of the stereospecific enzymatic hydrations of fumarate and *cis*-aconitate,[36] however, it was thought advisable to consider the stereochemical relationships between the α- and β-carbon atom substituents of *d*-isocitrate that are acted upon by aconitase. If the ring of natural isocitric acid lactone in which the α- and β-carboxyl are *cis* to each other is opened (FIGURE 6), it becomes immediately evident that the α-OH and β-H are stereochem-

This structure has recently been confirmed by means of crystallographic studies on naturally-occurring isocitrate by Glusker *et al.*[58]

In discussing the biochemical implications of the absolute configuration of natural *d*-isocitrate, Gawron *et al.*[54,55] have pointed out that such a structure is only compatible with a *trans* mechanism of addition of water across the double bond of *cis*-aconitate, and suggested a modification of the scheme proposed by Speyer and Dickman[53] for the mechanism of aconitase, taking into account the *trans* nature of the reaction. In accordance with the established absolute configuration of natural *d*-isocitrate, the aconitase-produced monodeuteroisocitrate is shown in FIGURE 6 in terms of the projection convention used in this paper. The original *cis*-aconitate proton and carboxymethyl group are *gauche* to one another, and such a structure is seen as arising by a *trans* mechanism of addition of D_2O across the double bond of *cis*-aconitate (FIGURE 5).

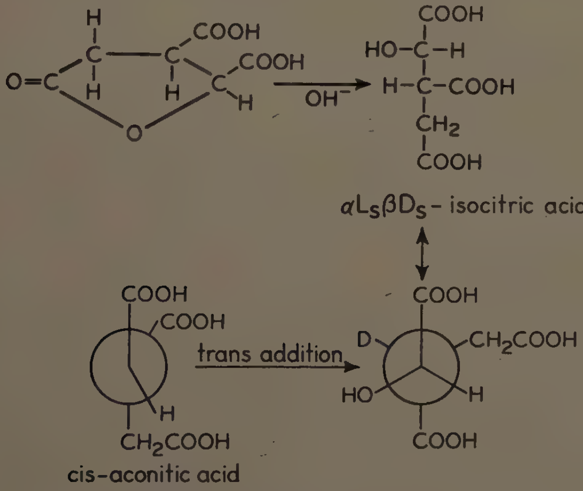

FIGURE 6. Absolute configuration of naturally-occurring dextrorotatory isocitric acid and steric course of the aconitase reaction (Gawron *et al.*[54,55]).

Origin of the Hydrogen Atom that Ultimately Appears in the Reduced Triphosphopyridine Nucleotide by Way of the Isocitric Acid Dehydrogenase Reaction

Experimental results. By taking advantage of the known specificity of the reactions in the tricarboxylic acid cycle, from fumarate to the generation of

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ically identical if one considers the α-carbon carboxyl group and hydrogen atom to be equivalent to the β-carbon carboxyl group and carboxymethyl group, respectively. Thus, when examined from a point outside the molecule on the axis joining the two asymmetric carbon atoms, the OH, COOH, and H groups attached to the proximal α-carbon atom, and the H, COOH, and CH_2COOH groups attached to the proximal β-carbon atom, will always appear in that equivalent order, reading clockwise. In subsequent discussions, therefore, the configuration of natural *d*-isocitrate was designated on the basis of the evidence brought forth[54-58] as OH-(αL), H-(βL)-isocitrate. Similar considerations were applied to the assignments of configuration to the deutero-L-malate isomers arising by either a *cis* or *trans* mechanism of addition of D_2O and H_2O to fumarate and dideuterofumarate, respectively, and to the various possible monodeuterocitrate isomers considered.

TABLE 4

REACTION OF ISOCITRIC ACID DEHYDROGENASE ON *D*-ISOCITRATE ARISING BY THE ACTION OF ACONITASE ON MONODEUTEROCITRATE ISOMERS DERIVED FROM STEREOSPECIFICALLY-LABELED DEUTERO-*L*-MALATE ISOMERS*

Experiment No.	Deutero-L-malate obtained by the action of fumarase on:	Compounds analyzed	Dilution factors	Deuterium content	
				Atom per cent excess†	Atom deuterium per molecule‡
1	Fumarate in D_2O	L-malic	16.9	0.33	1.00
		Citric	9.2	1.12	0.82
		Nicotinamide§	33.4¶	0.00	0.00
2	Dideuterofumarate in H_2O	L-malic	15.0	0.64	1.73
		Citric	17.4	0.61	0.85
		Nicotinamide§	38.6¶	0.29	0.67

In separate flasks, 24 ml. of 0.1 *M* fumarate in 0.05 *M* potassium phosphate (*p*H 7.7 in 99.8 per cent D_2O), and 24 ml. of 0.1 *M* dideuterofumarate in 0.05 *M* potassium phosphate (*p*H 7.4 in H_2O) were incubated at 28° C. with 270 units of crystalline fumarase (0.25 ml.). After reaching equilibrium, the reactions were stopped by heat inactivation and malic acid was separated on Dowex-1-formate columns by elution with continually-increasing formic acid concentrations. As a result, 1.87 mmoles each of 3-monodeutero-L-malate and 2,3-dideutero-L-malate were obtained. In preparation for the deuterium analyses, appropriate amounts of each isomer were diluted with normal L-malic acid and the malate samples were reisolated as the diphenacyl esters.

In separate flasks, 1643 μmoles each of 3-monodeutero-L-malic acid and 2,3-dideutero-L-malic acid, neutralized to *p*H 7.4, were then incubated with 5500 μmoles of tris(hydroxymethyl)amino-methane, *p*H 8.1; 2750 μmoles of KCl; 220 μmoles of $MgSO_4$; 550 μmoles of neutralized L-cysteine-HCl; 1650 μmoles of potassium pyruvate; 1375 μmoles of dilithium acetyl-phosphate; 37.4 μmoles of DPN^+; 1179 units of CoA; 263 units of lactic acid dehydrogenase; 1670 units of malic acid dehydrogenase; 386 units of transacetylase; and 4995 units of condensing enzyme in a total volume of 50.0 ml. The reactions were initiated by the addition of malic acid dehydrogenase, and the incubations were carried out at 28° C. The reactions were terminated by acidification to *p*H 1.1 with concentrated H_2SO_4. Citric acid determinations revealed the presence of 835 μmoles of citrate in the reaction mixture containing 3-monodeutero-L-malate, and 845 μmoles of citrate in the incubation mixture containing 2,3-dideutero-L-malate. The acidified reaction mixtures were extracted continuously with ether and the ether-extractable material chromatographed on Dowex-1-formate columns for the separation of citric acid. After appropriate dilution of each monodeuterocitrate isomer with normal citrate, a major portion of each sample was neutralized with dilute KOH to *p*H 7.5 to 7.8 for the subsequent reactions described below. From the remainder of each sample, on further dilution, citrate was isolated as the free acid by repeated recrystallizations from ethyl acetate and hexane, or from hot nitroethane.

For the final enzymatic transformations, each incubation mixture contained either 703 μmoles of diluted monodeuterocitrate derived from 3-monodeutero-L-malate or 721 μmoles of monodeuterocitrate synthesized from 2,3-dideutero-L-malate, plus 750 μmoles of glycylglycine, *p*H 7.4, in a total volume of approximately 13.5 ml. The reactions were initiated by the addition of 4 ml. of an aconitase solution (1850 units/ml.), preincubated at 0°C. in 5.1×10^{-4} *M* Fe $(NH_4)_2(SO_4)_2$ and 0.01 *M* cysteine, *p*H 7.4, for a minimum of 30 min. Additional 1.5-ml. portions of the same aconitase solution were added at 45, 90, 135, and 180 min. The aconitase equilibrations were carried out at 30° C. and extended over a total period of 225 min. At that time, each reaction mixture received 18 μmoles of $MnCl_2$, 93.4 μmoles of unneutralized TPN^+, and 9.8 units of isocitric acid dehydrogenase. The final volumes were adjusted to 30.0 ml.; the *p*H of each solution was found to be 6.7. When reduction of the added TPN^+ was essentially complete, the reactions were terminated by heat inactivation. Each of the solutions, after filtration, received 1.5 ml. of 0.1 *M* $K_3Fe(CN)_6$ and was incubated at 30° C. for 2 hours. The reaction mixtures were then adjusted to a final phosphate concentration of approximately 0.2 *M* at *p*H 9.4 and heated for 12 min. in a boiling-water bath. The nicotinamide samples were separated by column chromatography, properly diluted, and, after several recrystallizations, analyzed for deuterium.

* Data from Englard.[36]

† Experimental values.

reduced TPN by isocitric acid dehydrogenase, it is possible to determine the origin of the hydrogen atom appearing in the reduced nucleotide.[35,36] Two isomers of 3-deutero-L-malate were synthesized stereospecifically by the fumarase-catalyzed hydration of fumarate in 99.8 per cent D_2O and of dideuterofumarate (1.74 atoms of deuterium per molecule) in normal H_2O. Each of these isomers was incubated separately with malic dehydrogenase and DPN^+ (to form the corresponding isomers of monodeuterooxaloacetate); with pyruvate and lactic dehydrogenase (to regenerate oxidized DPN^+); with Coenzyme A (CoA), acetyl-phosphate, and phosphotransacetylase (to produce acetyl-CoA); and with citrate-condensing enzyme. The stereospecific synthesis of the two isomers of monodeuterocitrate was thus achieved. Each of the isomers was equilibrated with aconitase, and the corresponding *d*-isocitrates were subsequently oxidized with TPN^+ by isocitric acid dehydrogenase. The reduced nucleotides, subsequent to chemical reoxidation, were cleaved, and the isolated nicotinamide moieties were analyzed for their deuterium content. The results are summarized in TABLE 4.

Experiment 1 was initiated with 3-monodeutero-L-malate obtained by the equilibration of fumarate with fumarase in D_2O. The corresponding citrate contained 0.82 atom of deuterium per molecule; this value, representing an appreciable deviation from the theoretical value of 1.0, may partially reflect the degree of tautomerization of the intermediate monodeuterooxaloacetate and the experimental error in the quantitative determination of citrate before the addition of diluent citrate. On further treatment with aconitase, isocitric acid dehydrogenase, and TPN^+, this monodeuterocitrate isomer yielded unlabeled TPNH. Thus the α-deuterium atom of this citrate isomer, originally derived from D_2O in the fumarase reaction, must have been lost or transferred to the β-position in the formation of *d*-isocitrate by aconitase. In Experiment 2, the L-malate arising from the hydration of dideuterofumarate with fumarase in H_2O contained 1.73 atoms of deuterium per molecule. Since the α-carbon deuterium atom is lost in the formation of the intermediate oxaloacetate, the corresponding citrate contains roughly half of the deuterium initially present in the 2,3-dideutero-L-malate. The reduced TPN, arising by the enzymatic oxidation of the *d*-isocitrate corresponding to this citrate isomer with 0.85 atom of deuterium per molecule, contained 0.67 atom of deuterium per molecule after chemical oxidation to TPN^+ with neutral ferricyanide. It is therefore evident that the α-deuterium atom of this citrate isomer, originally derived from the α or α'-carbon-bound deuterium of dideuterofumarate, was not involved in the reversible dehydration reaction catalyzed by aconitase. The corresponding *d*-isocitrate of this monodeuterocitrate isomer must therefore have contained 0.85 atom of deuterium per molecule bound to the α-carbon

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TABLE 4—(*Continued*)

‡ Values corrected for dilution and calculated on the basis that one atom of deuterium per molecule of diphenacyl-malate, citric acid, and nicotinamide would correspond to a value of 5.55, 12.5, and 16.7 atom per cent excess, respectively.

§ The deuterium content of the nicotinamide is identical to the deuterium content of the oxidized TPN^+ from which it was derived.

¶ The dilution factors for the nicotinamide samples also include the initial dilution of the citrate isomers prior to aconitase equilibration. These initial dilutions differ from the dilution factors recorded for the corresponding citric acid samples analyzed for deuterium (see legend).

atom which, on subsequent direct transfer to TPN^+, theoretically yielded TPND with the same isotope concentration. The enzyme isocitric acid dehydrogenase has recently been shown to have steric specificity for the α side of the pyridine ring.[59] α-DPND retains about 50 per cent of its deuterium after chemical oxidation to DPN^+ with neutral ferricyanide,[60] whereas chemical oxidation of β-DPND leads to an almost quantitative retention of the isotope in the oxidized nucleotide.[51] In view of these considerations, the α-TPNH of Experiment 2 with a theoretical isotope content of 0.85 atom of deuterium per molecule should have yielded approximately 0.43 atom of deuterium per molecule in the reoxidized nucleotide. The reasons for the excessive retention of isotope in the TPN^+ of Experiment 2 are not clear. It is possible, however, that the chemical reoxidation of α-TPND may result in a more quantitative deuterium retention in the oxidized nucleotide than is the case with the oxidation of α-DPND.

Stereochemical implications of the results as regards the mechanism of the fumarase reaction. As previously mentioned, the conclusion of a *cis* fumarase mechanism rested on nuclear magnetic resonance measurements interpreted on the basis of an assumed specific configuration for L-malic acid in the solid state.[50] Since doubts were subsequently expressed about the correctness of this assumption[61] it was felt that the problem of a *cis* versus *trans* fumarase mechanism could be resolved from the experiments just described, designed to establish the configuration of the product of the stereospecific enzymatic hydration of fumarate in D_2O in relation to the stereospecificity of the aconitase-catalyzed reaction.

The stereochemical consequences of the experimental approach to this problem may be visualized by an analysis of the reaction sequences formulated in FIGURE 7. Reaction **1** illustrates the fumarase-catalyzed hydration of fumarate in D_2O. In view of the demonstrated stereospecificity of this reaction,[31-33] occurring by either a *trans* or *cis* mechanism of addition, only one possible isomer of 3-monodeutero-L-malate is obtained. Reaction **2** represents the oxidation of 3-monodeutero-L-malate to monodeuterooxaloacetate by malic dehydrogenase in the presence of DPN^+. Since oxaloacetate has been shown to be enzymatically reduced in the keto form[62,63] and therefore probably also arises as keto oxaloacetate from L-malate in the reverse reaction, the stereochemistry of the β-carbon is unaffected. The condensation of monodeuterooxaloacetate with acetyl-CoA is described in Reaction **3**. It has recently been shown that keto oxaloacetate is the substrate of the citrate condensing enzyme[64] and therefore, the stereochemistry of the α-carbon of monodeuterocitrate (depicted as projecting behind the plane of the paper) derived from the β-carbon of monodeuterooxaloacetate (or from the initial 3-monodeutero-L-malate), remains unchanged. Some racemization (and loss of deuterium) may occur by way of the reversible nonenzymatic keto-enol tautomerization of the monodeuterooxaloacetate intermediate. The extent of racemization (or loss of deuterium) thus will depend on the relative rates of this tautomerism and the condensation reaction with acetyl-CoA. It was therefore necessary, for the purpose of the experimental approach used, to generate slowly the monodeuterooxaloacetate by limiting the amount of malic dehydrogenase, and to maintain high rates of citrate synthesis by using an excess of condensing enzyme and a large amount of phosphotransacetylase to regenerate rapidly acetyl-CoA from acetyl-phosphate in the presence of CoA. Since no alterations have occurred in the asymmetry of the monodeuterocitrate α-carbon derived from the original β-carbon of 3-monodeutero-L-malate by way of Reactions **2** and **3**, the absolute configuration of the α-carbon of the monodeuterocitrate isomer actually obtained by the sequence of reactions listed here must be determined by the mechanism of the fumarase reaction. Obviously, if the sequence of reactions illustrated in FIGURE 7 were initiated by the fumarase catalyzed hydration of dideuterofumarate in normal water, the α-carbon configurations of the resulting possible monodeuterocitrate isomers will be the reverse of the corresponding isomers listed here.

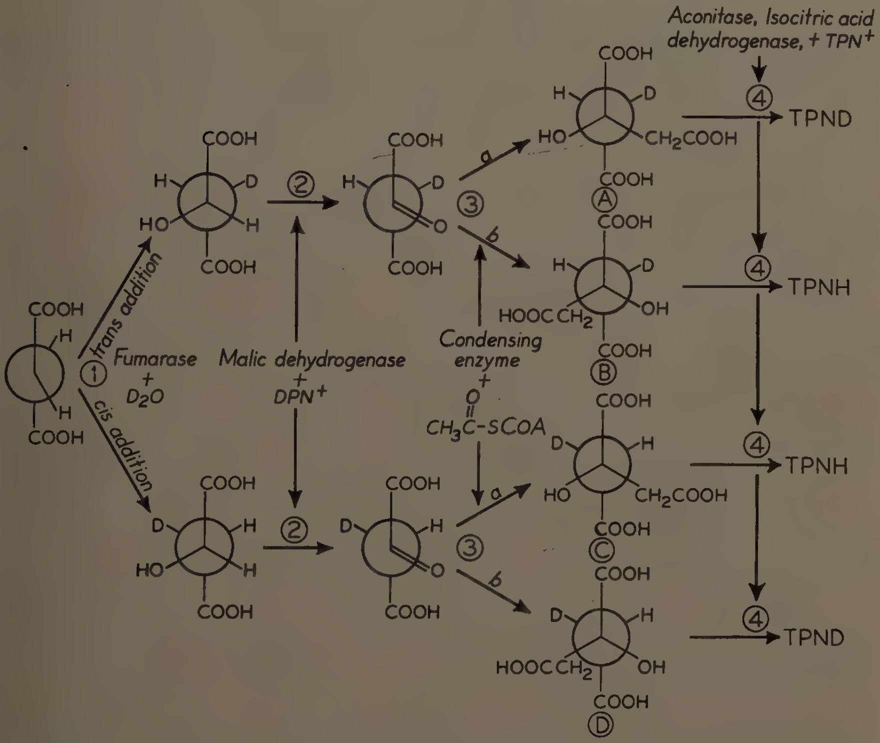
Aconitase, Isocitric acid dehydrogenase, + TPN+
④
TPND
TPNH
TPNH
TPND
trans addition
cis addition
①
Fumarase + D2O
Malic dehydrogenase + DPN+
②
③
α
β
Condensing enzyme + O=CH3C-SCOA
COOH
H
D
HO
OH
O
CH2COOH
HOOCCH2
Ⓐ
Ⓑ
Ⓒ
Ⓓ

FIGURE 7. Stereospecific synthesis of monodeuterocitric acid, and stereochemical considerations relative to the origin and fate of possible monodeuterocitric isomers (Englard[36]).

NOTE: Using the arbitrary nomenclature previously proposed,[36] the products of the *trans* and *cis* addition of D_2O to fumarate have been designated as OH-(αL), deuterium-(βD)-malic acid, and OH(αL), deuterium-(βL)-malic acid, respectively. Similarly, the following assignments of configuration have been made to the monodeuterocitrate isomers: A, deuterium-(αD), OH-(βL); B, deuterium-(αD), OH-(βD); C, deuterium-(αL), OH-(βL); and D, deuterium (αL), OH-(βD).

The inclusion of citrate as an intermediate in the tricarboxylic acid cycle necessitates the postulation that this acid reacts asymmetrically in both its synthetic and degradative pathways.[1-6] Hence, as regards the specific configuration of the β-carbon of the enzymatically-synthesized monodeuterocitrate, only one of the two possibilities indicated as arising in FIGURE 7 by either Reactions **3*a*** or **3*b*** may exist. The OH group on the β-carbon of the product of Reaction **3*a*** occupies the same spatial position as the OH group on the α-carbon of L-malate, whereas the configuration of the β-carbon of the citrate arising by way of Reaction **3*b*** is opposite to that of the α-carbon of L-malate. Thus of the four possible isomers listed in FIGURE 7 (which incidentally correspond to the four possible configurations shown earlier in FIGURE 5 as arising by either a *cis* or *trans* addition of D_2O to *cis*-aconitate), only one isomer will be obtained by the sequence of Reactions **1** to **3**, and its absolute configuration will be dictated by the mechanism of the fumarase reaction and the steric course of the citrate condensation reaction.

We may now proceed to discuss the relevance of Reaction **4** to the stereochemical relationships just described. Since a *trans* mechanism for the reversible dehydration of isocitrate by aconitase[54,55] also implies a *trans* mechanism for the reversible dehydration of citrate by the same enzyme, one can predict whether a hydrogen or deuterium atom is eliminated from the appropriate methylene group of each of the indicated monodeuterocitrate isomers. To avoid the necessity of isolating *cis*-aconitate, which would present technical problems because of its low concentration at equilibrium,[65-68] one can take advantage of another previously-described observation.[34] Thus, since the α-hydrogen or deuterium not involved in the aconitase reaction ultimately find its way into the reduced TPN through the isocitric acid dehydrogenase reaction, one can further predict whether TPNH or TPND is obtained from any one of the four possible isomers of monodeuterocitrate. As an example, let us consider the fate of the α-deuterium atom of isomer A. This structure, when subjected to a *trans* mechanism of water elimination, will lose the α-hydrogen atom to yield monodeutero-*cis*-aconitate. On rehydration, the isocitrate will now contain in the α-position the deuterium atom that did not participate in the aconitase-catalyzed dehydration of the specific monodeuterocitrate isomer under consideration. This monodeuteroisocitrate will yield TPND through the direct hydrogen transfer reaction catalyzed by isocitric acid dehydrogenase.

It is therefore apparent that, as a result of the specificity of each one of the steps involved, initiating the reactions with unlabeled fumarate in D_2O results in the formation of either TPNH or TPND. However, as indicated in FIGURE 7, the presence of deuterium in the reduced TPN is compatible with either of two structures of monodeuterocitrate, which in turn reflect opposite combinations as regard the steric courses of the fumarase and citrate condensation reactions. Thus, in order to interpret the final results in terms of an unequivocal *cis* versus *trans* fumarase addition mechanism, the steric course of the reaction catalyzed by the citrate condensing enzyme must be known.

An insight into this problem may be obtained by considering the implications of the results obtained by Martius and Schorre,[6,69] partially summarized in FIGURE 8. These investigators described the synthesis of oxalocitramalic acid lactone by the aldol condensation of oxaloacetic and pyruvic acids. The enantiomorphs of oxalocitramalic acid lactone, upon resolution by way of the brucine salts, were incubated in D_2O to achieve the complete exchange of the hydrogen atoms on the methylene carbon atom adjacent to the carbonyl group and subsequently oxidized with D_2O_2 to yield each of the two enantiomorphs of dideuterocitric acid separately.[69] Application of Hudson's rule permitted assignment of absolute configuration to the enantiomorphs of oxalocitramalic acid lactone and, hence, also to the corresponding dideuterocitric acid isomers. As shown in FIGURE 8, on enzymatic degradation the α-ketoglutarate derived from the L (−) isomer retained all the deuterium, whereas that from the D (+) isomer, not shown here, contained no deuterium.[6] It is then possible to assign a configuration to natural citrate in the sense that the aconitase inactive carboxymethyl group known to be derived from acetyl-CoA can be specified.

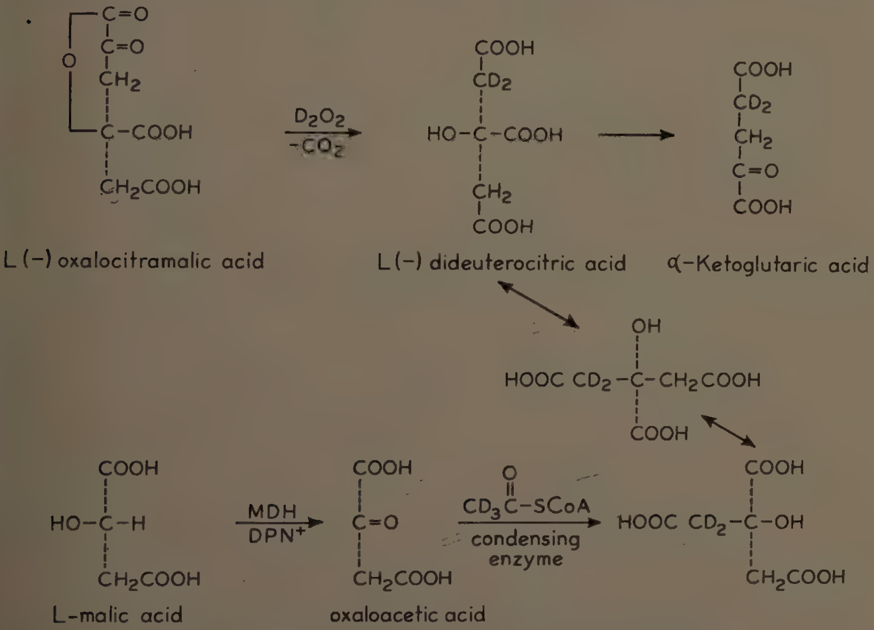

FIGURE 8. Steric course of the citric acid-condensing enzyme.

The structure of the L (−) isomer has been formulated by Schwartz and Carter[10] as shown, with the OH "up" and the COOH "down" and both projecting behind the plane of the paper. The carboxymethyl group on the right is then the oxaloacetate-derived aconitase-active group, and the CD_2COOH group on the left originates from acetyl-CoA and represents the aconitase-inactive group in biologically-synthesized citrate. This very same structure, as indicated, can be rewritten in terms of the more conventional Fisher projection with the carboxyl group on top, the R group, in this case the oxaloacetate-derived carboxymethyl group, on the bottom, and both projecting behind the plane of the paper. A comparison of the absolute configuration of the β-carbon of enzymatically-synthesized citrate with that of the α-carbon of L-malate then reveals that the steric course of the condensation reaction results in a structure in which the OH group on the β-carbon occupies a spatial position opposite to that of the OH group on the α-carbon of L-malate. This conclusion is predicated on the correctness of Hudson's rule as applied to the enantiomorphs of oxalocitramalic acid lactone, and it is therefore pertinent to consider what conclusions can be reached as regards the steric courses of analogous biological condensation reactions from the known specificity of the enzymes involved for a particular optical isomer. Thus the malate synthesase reaction formulated in FIGURE 9, in order to conform to the known specificity of the enzyme for L-malate,[70] must also be described as occurring by placing the carboxymethyl group on the left and the OH group on the right in the condensation of acetyl-CoA with glyoxylic acid. The enzyme isocitratase,[71] catalyzing the reversible condensations of succinic acid with glyoxylic acid as described in FIGURE 10, when analyzed in a similar manner is shown to proceed in an analogous fashion

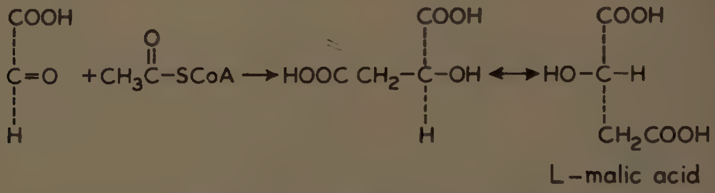

FIGURE 9. Steric course of malate synthesase.

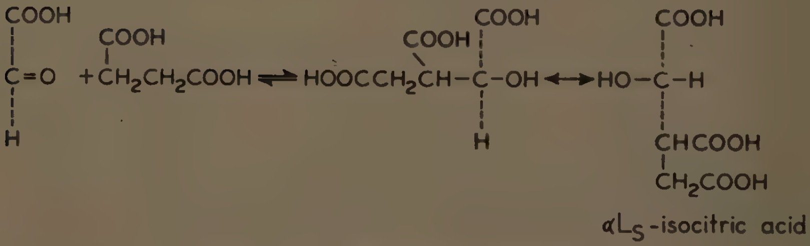

FIGURE 10. Steric course of isocitritase.

in order to obtain a structure compatible with the known absolute configuration of the α-carbon atom of naturally occurring dextrorotatory isocitrate.

Returning once again to FIGURE 7, since the evidence brought forth indicates that the steric course of the condensation reaction is compatible only with Reaction **3*b***, starting with fumarate and D_2O, a *trans* mechanism of fumarate hydration will yield TPNH and a *cis* mechanism must result in the formation of TPND. As mentioned before, initiation of the reaction sequence with dideuterofumarate in normal water results in citrate α-carbon configurations opposite to those recorded here, so that a *trans* fumarase mechanism would result in the production of TPND and a *cis* mechanism should yield TPNH. As summarized in TABLE 4, the previously discussed results, in conjunction with the stereochemical relationships just discussed, therefore point to a *trans* fumarase addition mechanism.

The validity of this latter conclusion, which now completes our knowledge of the stereochemistry of the reactions from fumarate to isocitrate, may be examined in terms of some further consequences of the stereochemical restrictions imposed by the specificity of the aconitase reaction. It has been suggested by Krebs[72] that in the hydration of fumarate, if one assumed a three-point attachment so that only one member of the double bond was attacked, one could account for the optical specificity of the fumarase reaction. As shown in FIGURE 11 under *A*, whether the OH group adds to C_1 or C_2 of fumarate from the same direction, the absolute configurations of the hydroxylated C_1 and C_2 are identical. Ogston[73] has pointed out that similar considerations account for the optical specificity of the aconitase reaction with respect to *d*-isocitrate and for the formation of both citrate and isocitrate from *cis*-aconitate by the action of a single enzyme. Thus, depending on whether the OH group adds to C_1 or C_2 of *cis*-aconitate, one obtains isocitrate or citrate respectively (FIGURE 11*B*). There is, however, a stereochemical consequence that results from the fact that the aconitase, unlike the fumarase reaction, causes the hydration of a *cis* double bond. As illustrated under *B* in FIGURE 11, the addition of OH to C_1 or C_2 of *cis*-aconitate from the same direction to the same component of the double bond leads to stereochemically-opposite hydroxylated carbon atoms. In view of the demonstrated intramolecular hydrogen shift in the direct interconversion of *d*-isocitrate to citrate,[53] it is likely that the same enzymatic site is involved in the addition of hydrogen to *cis*-aconitate to form citrate and isocitrate. One may also consider a similar identity of the OH donating en-

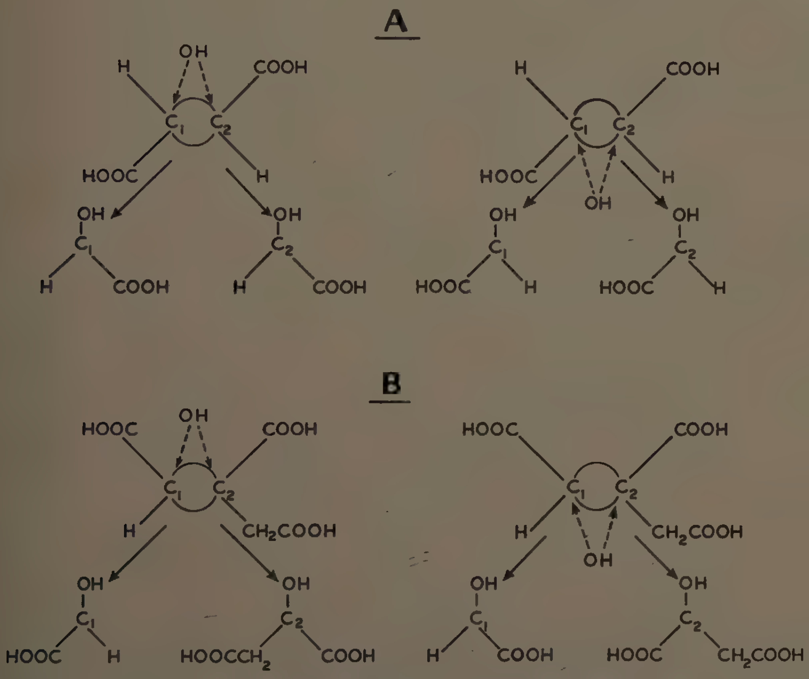

FIGURE 11. Steric consequences arising from a preferred direction of addition of OH to either of the unsaturated carbon atoms of fumaric and *cis*-aconitic acids.

zymatic site. Thus, in accordance with Ogston's proposal,[73] it is suggested that the same sites adding either the OH group or H atom to the asymmetric enzyme-*cis*-aconitate intermediate in the formation of optically active *d*-isocitrate, also add from the same direction to the opposite carbon atoms in the formation of citrate. Such a mechanism must necessarily lead to opposite configurations of the respective α- and β-carbons of citrate and isocitrate. This stereochemical description of the aconitase reaction is in agreement with the proposed mechanism of aconitase action as presented by Gawron *et al.*[65] In this latter paper the *trans* stereochemistry in going reversibly from *cis*-aconitate to isocitrate was established by ascertaining the absolute configuration of naturally occurring *d*-isocitrate. A similar reversible *trans* mechanism of citrate dehydration, through a protonated *cis*-aconitate common intermediate, was then depicted in FIGURE 6 of Gawron *et al.*'s paper to yield a citrate molecule with α- and β-carbon configurations opposite to those of the corresponding carbon atoms in *d*-isocitrate.

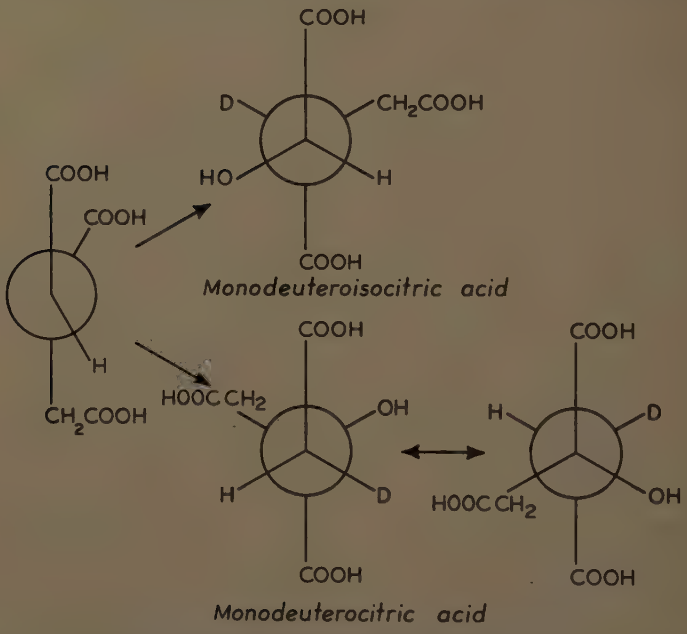

FIGURE 12. Predicted absolute configuration of monodeuterocitric acid resulting from aconitase-catalyzed *trans* addition of D_2O to *cis*-aconitic acid.

As indicated in FIGURE 12, since the absolute configuration of *d*-isocitrate has been shown to correspond to the structure of monodeuteroisocitrate depicted here, the corresponding monodeuterocitrate must adhere to the absolute configuration indicated below it. To conform with the previously-discussed projection formulas in FIGURE 7, this structure of monodeuterocitrate can be rewritten as shown with the α-carbon now projecting behind the plane of the paper. The absolute configuration of this monodeuterocitrate is seen to be equivalent to that of isomer B in FIGURE 7. The β-carbon configuration is in agreement with the previously-discussed prediction based on the work of Martius and Schorre.[6,69] The α-carbon configuration is equivalent to that of the β-carbon configuration of 3-monodeutero-L-malate arising specifically from a *trans* mechanism of D_2O addition to fumarate, as catalyzed by the enzyme fumarase. In retrospect, by considering all the implications of what was known about the aconitase reaction, only a *trans* fumarase mechanism could have accounted for the observation that the α or α′ carbon-bound hydrogen or deuterium of fumarate eventually appears in the reduced TPN through the reactions represented in this partial sequence of the tricarboxylic acid cycle.

The conclusions reached from the results presented here, based on configurational considerations in relation to the mechanism of the stereospecific enzymatic hydration of fumarate and *cis*-aconitate, have been fully confirmed by independent means. The validity of the proposed experimental approach, which with certain appropriate modifications can be applied to other systems to resolve similar problems, has therefore been firmly established.

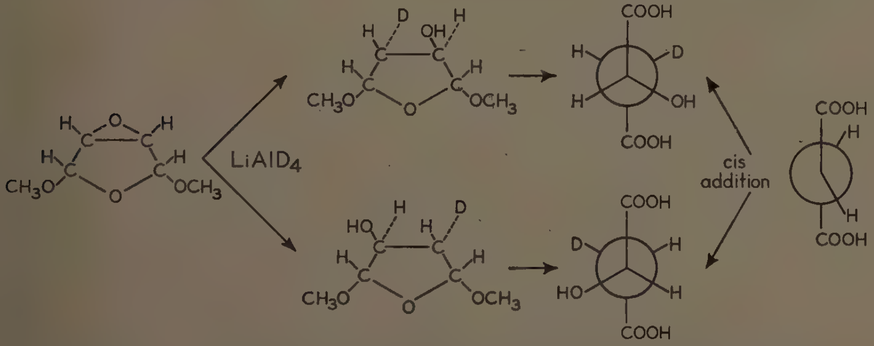

FIGURE 13. Stereospecific chemical synthesis of 3-monodeutero-DL-malic acid.

Further Evidence for the trans Nature of the Fumarase Reaction

The *trans* fumarase mechanism has recently been confirmed by an independent synthetic approach. Thus, as shown in FIGURE 13, Gawron and Fondy[74] and, more recently, Anet[75] were able to achieve a stereospecific synthesis of 3-monodeutero-DL-malate by the *trans* opening of the oxide ring of 3,4-epoxy-2,5-dimethoxytetrahydrofuran with lithium aluminum deuteride, followed by acid hydrolysis and subsequent oxidation of the dialdehyde to the corresponding hydroxydicarboxylic acid. With the α- and β-carbon carboxyl groups in a *trans* configuration, the structures of the two enantiomorphs, resulting from the synthetic approach used, must be represented with the two protons as being *gauche* to each other.* In addition, such configurations are shown to arise from fumarate only by way of a *cis* mechanism of D_2O addition (FIGURES 3 and 7). The high-resolution nuclear magnetic resonance spectrum of this synthetic racemate was different from that obtained with the product of the enzymatic stereospecific hydration of fumarate in D_2O. The coupling constant of 4.4 ± 0.2 cps[74] obtained with the synthetic racemate, however, was similar to that obtained by Krasna[30] with 3-monodeutero-D-malate prepared from enzymatic 3-monodeutero-L-aspartate by the reactions outlined in FIGURE 14. The first reaction describes the aspartase-catalyzed stereospecific amination of fumarate in D_2O.[29,30] Here again, two possible isomers are depicted, depending on the steric course of the reaction. Treatment with nitrosyl bromide, a reaction occurring with complete retention of configuration,[76] yields 3-monodeutero-

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* The enantiomorphs have been referred to as α-OH_{L_s}–β-deuterium$_{D_s}$–malic acid and α-OH_{D_s}–β-deuterium$_{L_s}$–malic acid by Gawron and Fondy,[74] using their earlier-proposed system of nomenclature.[54,55] Anet[75] refers to these enantiomorphs as *threo*-3-deutero-L-malic acid and *threo*-3-deutero-D-malic acid, respectively. In terms of the arbitrary nomenclature previously used by Englard,[36] the enantiomorphs would be designated as OH-(αL), deuterium-(βL)-malic acid and OH-(αD), deuterium-(βD)-malic acid, respectively.

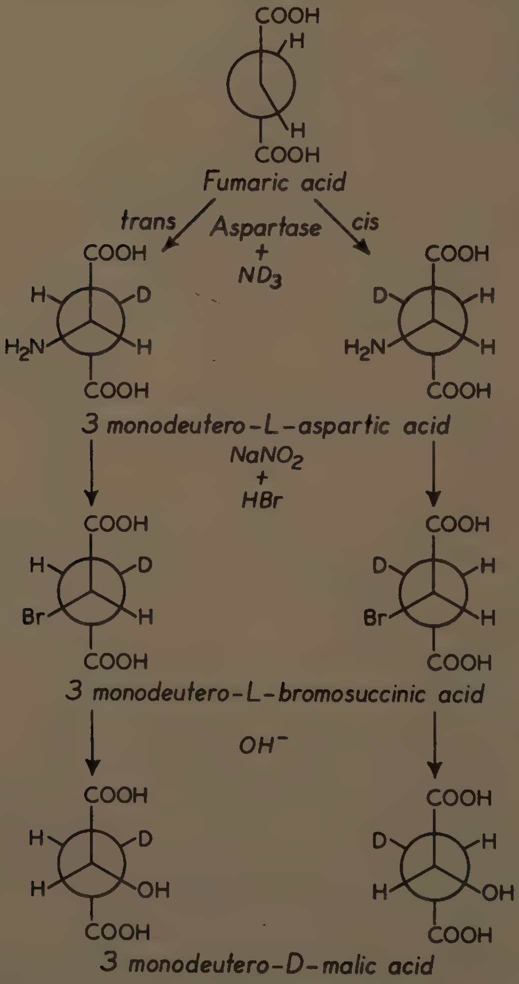

FIGURE 14. Stereospecific conversion of aspartase-produced 3-monodeutero-L-aspartic acid to 3-monodeutero-D-malic acid.

L-bromosuccinate. The subsequent displacement reaction by means of hydroxide ion in methanol, which occurs with complete inversion of configuration,[77] yields 3-monodeutero-D-malate. In all these reactions the stereochemistry of the β-carbon remains unchanged. Since the 3-monodeutero-D-malate obtained from the product of the stereospecific enzymatic amination of fumarate in D_2O by the reaction steps just described, has the same coupling constant as the stereospecifically-labeled synthetic racemate,[74] its configuration must be such that the two protons are also *gauche* to each other. Such a configuration for the 3-monodeutero-D-malate could only have arisen by the sequence of reactions outlined in FIGURE 14 from an initial *trans* aspartase mechanism. The product of the aspartase reaction (L-aspartate) has the same spatial configuration with respect to the asymmetric α-carbon atom as the product of the fumarase reaction (L-malate). In addition, other studies by Krasna[30] and Englard[29] have shown that both enzymes catalyze a deuterium addition specifically to one and the same position on the methylene group of L-malate or L-aspartate. The conclusion must therefore be reached that the fumarase reaction also proceeds by a *trans* mechanism of addition of water to the double bond of fumarate.

Finally, in a recent preliminary note,[78] the *p*H dependence of the competitive inhibition of fumarase by succinate and the three isomers of tartrate have been studied with L-malate as the substrate. Although no data are available at the present time, it was indicated that the results were interpreted in terms of a steric model for the active site, based on a *trans* mechanism for the dehydration of L-malate by fumarase.

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EFFECTS OF D_2O ON CELLULAR COMPONENTS OF MAMMALIAN CELLS GROWN IN TISSUE CULTURE*

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The effects of deuterium oxide concentration on cells grown in tissue culture were first studied by Fischer in 1936,[1] and additional observations have been recorded by Cagianut,[2] Siegel,[3] and by ourselves (elsewhere in these pages). In general, it has been observed that cells can survive in deuterium oxide concentrations as high as 50 per cent, deterioration and death occurring at higher concentrations.

While earlier investigations were centered around cytologic changes which appeared in deuterated cells, none were concerned with the effects of deuterium oxide on cell composition. Our observations‡ that incorporation of deuterium oxide into the medium of HeLa cells caused cell enlargement and the appearance of lipid-containing vacuoles prompted this investigation. The influence of deuterium oxide on the growth of tissue culture cells has already been described elsewhere.‡ We chose to work at a concentration of 20 per cent deuterium oxide, which permitted cell growth but at which various effects of deuteration were already clearly discernible.

Methods

Three lines of tissue culture cells were used in this work, HeLa, L, and 5178. The first and second cell lines were grown in stationary culture and the third in suspended culture. Details concerning the growth media and methods of culture are described elsewhere.‡ Deuterium oxide (D_2O) was added to medium concentrates, and water was also added to adjust the final volume. The medium was sterilized by filtration, using conventional techniques.

Cells grown for specified periods of time were harvested, washed, and weighed in the wet and lyophilized states. The acid-soluble fraction was obtained by treating the cell powder with 10 per cent $HClO_4$ for 10 min. at 4°C. and centrifuging the mixture at from 2000 to 3000 g for 10 min. at 1° C. Lipids were extracted from the cell powder with ethanol-ether 2:1 for 24 hours in a Soxhlet apparatus. The extract was taken to dryness under reduced pressure and redissolved in petroleum ether for subsequent chromatographic separation on silicic acid columns.[4]

Glycerides were detected using the D. A. Turner modification (personal communication) of the Zilversmit test.[5] Phosphorus was determined by the method of Fiske and Subbarow,[6] ribose compounds by the method of Mejbaum,[7] and deoxyribose compounds by the method of Dische and Schwarz.[8] Sterols were determined by the method of Trinder,[9] and free fatty acids by titration with standard methanolic alkali.

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‡ L. A. Manson, E. I. Carp, V. Defendi, E. L. Rothstein, R. W. Hartzell, Jr., D. Kritchevsky, this monograph.

Results and Discussion

Not only does D_2O cause changes in the pattern of growth, but also in the size of the cell and its water content. As can be seen in TABLE 1, the 5178 cell probably accumulates water with aging, but does so even more when grown in the presence of D_2O. It is also evident that cells grown in suspended culture became larger than did control cells. TABLE 2 shows the results of a typical experiment with L cells. In this instance, as before, cells exposed to D_2O contained proportionately more water. The figures also suggest that the deuterated cell may be larger by virtue of increased water content and yet contain fewer solids than control cells. In the case of the HeLa cell (TABLE 3), the deuterated cells appear to be slightly larger than control cells, but not because of increased water content. It should also be noted that increased burst sizes for poliovirus have been observed in deuterated HeLa cells.* We have also reported an increased burst size for bacteriophage T7 in deuterated *Escherichia coli*, strain B, which also appeared to be larger than nondeuterated cells.[10]

TABLE 1

EFFECTS OF D_2O ON THE SIZE AND WATER CONTENT OF 5178 CELLS

Medium	Days grown	Wet wt.:dry wt.	Wet wt. (mg./10^6 cells)	Dry wt. (mg./10^6 cells)
H_2O	3	7:1	0.625	0.087
D_2O	3	12:1	0.833	0.070
H_2O	5	11:1	0.769	0.067
D_2O	5	16:1	0.667	0.043

TABLE 2

EFFECTS OF D_2O ON THE SIZE AND WATER CONTENT OF L CELLS
(Grown 4 days)

Medium	Wet wt.:dry wt.	Wet wt. (mg./10^5 cells)	Dry wt. (mg./10^5 cells)
H_2O	11:1	0.769	0.067
D_2O	21:1	1.111	0.053

TABLE 3

EFFECTS OF D_2O ON THE SIZE AND WATER CONTENT OF HELA CELLS

Medium	Days grown	Wet wt.: Dry wt.	Wet wt. (mg./10^5cells)	Dry wt. (mg./10^5 cells)
H_2O	3	Lost	—	—
D_2O	3	8:1	0.286	0.037
H_2O	5	8:1	0.333	0.042
D_2O	5	7:1	0.370	0.050

* L. A. Manson, E. I. Carp, V. Defendi, E. L. Rothstein, R. W. Hartzell, Jr., D. Kritchevsky, this monograph.

Examination of the acid-soluble contents of the three lines of cells exposed to D_2O allow the generalization to be made that inorganic phosphorus, organic phosphorus, and ribose-containing compounds are progressively lost from deuterated cells. Work is now in progress which will attempt to elucidate these changes.

Examination of the lipid composition of the three cell lines under investigation showed certain differences among them. It may be seen (TABLE 4) that in the case of 5178 cells aging apparently causes a decrease in the phospholipid content of these cells. Exposure of the cells to D_2O-containing media caused a still greater loss. In this particular instance, two days' aging caused a 13 per cent decrease, while the deuterated cells showed a loss of 52 per cent over the same time period. Significant increases in total glyceride are also observed. Preliminary analysis indicates that an increase in triglyceride is primarily responsible for this increment. Somewhat similar results may be seen for L cells exposed to D_2O (TABLE 5). There is a decrease in phospholipid content and an increase in total glyceride. This increase in total glyceride seems, again, to be attributable to triglyceride. An increase in esterified

TABLE 4

EFFECTS OF D_2O ON THE LIPID CONTENT OF 5178 CELLS

	Days grown in D_2O*			
	3		5	
	Control	D_2O	Control	D_2O
Phospholipid P	0.31	0.28	0.27	0.16
Total glyceride	1.9	3.2	2.1	3.3
Free sterol	0.33	0.57	0.34	0.21
Esterified sterol	0.07	0.12	0.27	0.24
Total sterol	0.40	0.69	0.61	0.45
$\frac{\text{Esterified sterol}}{\text{Free sterol}}$	0.21	0.21	0.80	1.1

* All figures given as micrograms/10^6 cells.

TABLE 5

EFFECTS OF D_2O ON THE LIPID CONTENT OF L CELLS

	Control*	Grown 4 days in D_2O*
Phospholipid P	2.3	2.0
Total glyceride	70.0	125.0
Free sterol	27.4	26.0
Esterified sterol	9.7	14.5
Total sterol	37.1	40.5
$\frac{\text{Esterified sterol}}{\text{Free sterol}}$	0.35	0.56

* All figures given as micrograms/10^6 cells.

sterol is also seen. TABLE 6 shows the results with the HeLa cell. An increase in free and esterified sterol is observed. Although the total glyceride content of control and experimental cells is similar, preliminary investigations on the glyceride composition indicate an increased triglyceride content in D_2O-grown cells.

Weinhouse[11] has shown that the endogenous respiration of the cell depends, more often than not, on the oxidation of fatty acids. Since changes in the glyceride content of cells must ultimately reflect differences in the metabolism of fatty acids, a few experiments with HeLa cells were performed to ascertain the effects on the respiration of the cell. The Q_{O_2} and the Q_{CO_2} for normal and deuterated HeLa cells were determined. The normal or deuterated cells were suspended in water or 50 per cent D_2O-containing buffers. These experiments, using conventional manometric procedures for the Warburg apparatus, were allowed to run for 90 min.

TABLE 6
EFFECTS OF D_2O ON THE LIPID CONTENT OF HELA CELLS

	Days grown in D_2O*			
	2		4	
	Control	D_2O	Control	D_2O
Phospholipid P	—	1.5	1.8	1.5
Total glyceride	—	21.5	21.0	—
Free sterol	—	0.43	0.36	0.51
Esterified sterol	—	5.1	3.4	4.2
Total sterol	—	5.53	3.76	4.71
$\frac{\text{Esterified sterol}}{\text{Free sterol}}$	—	11.8	9.5	8.3

* All figures given as micrograms/10^6 cells.

The most striking features observed were the following:

(1) The suspension of normal or deuterated cells in D_2O-containing buffers resulted in an increased Q_{CO_2} over that observed for the control system, the mean values being 3.8 for normal cells suspended in H_2O buffers and 5.4 for normal cells in D_2O buffers. Deuterated cells suspended in D_2O buffers displayed a value of 4.4.

(2) The exposure of normal or deuterated cells to D_2O buffers resulted in a cancellation of the Crabtree effect usually observed with HeLa cells. In other words, the addition of glucose to cells suspended in D_2O buffers did *not* result in a decreased Q_{O_2}.

(3) The addition of palmitic, stearic, or oleic acid to a concentration of 0.005 *M* proved to be toxic in terms of O_2 uptake. The presence of D_2O in the suspending buffer partially relieved this inhibition.

Other investigators[12,13] have studied the effects of metabolic inhibitors on the lipid composition of L cells. In the experiments cited, the total lipid was not studied and results were presented as micrograms lipid per milligram cell protein. Of the observations pertinent to our work, Cailleau *et al.*[12] found that iodoacetate caused an increase in total fatty acid but none in lipid phosphorus, while azide caused an increase in both components. They found dinitrophenol to have no effect on the two lipid fractions studied. King and his co-workers[13] studied the influence of *p*-fluorophenylalanine and the cholesterol/protein ratio and found an increased cholesterol content in treated cells. Our data indicate that in L cells, D_2O causes increases in glyceride and sterol ester content, which would explain both the increases in fatty acid and cholesterol observed by others and our own observation that lipid phosphorus was not increased.

These preliminary observations are provocative, but considerable subsequent study is indicated before any definite assessment of the role of D_2O in lipid synthesis and utilization can be made. Rabinowitz and Kritchevsky (J. L. Rabinowitz and D. Kritchevsky, this monograph) have demonstrated enhancement of lipogenesis by mouse liver homogenates containing 75 per cent D_2O. It is well known that fat accumulates in old, injured cells. The accumulation of lipid in such cells has been termed fatty degeneration or infiltration, depending upon whether it is regarded as unmasking of intracellular lipid or deposition from sources outside the cell. The system that we have described may, with further elaboration and the use of isotopically labeled precursors, yield clues to the mechanisms of these fatty changes.

TABLE 7

EFFECTS OF D_2O ON CELLULAR COMPONENTS OF MAMMALIAN CELLS

	5178	Cell Line* L	HeLa
Cell size	+	+	+
Inorganic P	−	±	−
Organic P	−	−	−
Ribose compounds	−	−	−
Phospholipid P	−	−	−
Total glyceride	+	+	+
Free sterol	−	−	±
Esterified sterol	±	±	+

* Decrease, −; increase, +; questionable ±.

The enlargement of the cells grown in D_2O is similar in some respects to the giant cells produced by irradiation.[14] The giant cells' increased size is apparently related to the continued synthesis of macromolecules incapable of dividing. Such cells are still capable of synthesizing virus.[15] It is of interest to note that irradiation also causes enhancement of lipid synthesis.[16,17] Studies of the similarities and differences between these two systems (D_2O and irradiation) offer a new and stimulating area for research.

Summary

Our findings suggest that D_2O presents a new and useful tool for the study of certain aspects of lipid metabolism and are summed up in TABLE 7.

In respirometry experiments with HeLa cells, deuterium oxide caused an increased Q_{CO_2} and a reversal of the inhibition of the oxygen uptake normally seen with glucose.

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HEPATIC LIPOGENESIS IN D_2O-FED MICE*

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While high concentrations of deuterium are toxic to mammals and higher plants,[1] moderate levels of deuteration are tolerated for considerable lengths of time,[2] the most readily observed effects being hyperexcitability and hypermetabolism.[3-6] When mammalian cells are maintained in tissue culture media containing 25 to 50 per cent D_2O, they become enlarged and vacuolized and show some nuclear degeneration.[7-9] We have observed that the vacuoles are lipid-containing[8] (elsewhere in these pages), and we have also seen that changes in cellular lipids occur in mammalian cells grown in media containing D_2O (this monograph). In view of these past observations relating some aspect of the D_2O effect to lipid metabolism, we were prompted to investigate the effects of D_2O feeding on hepatic lipogenesis.

Materials and Methods

Male Swiss mice were maintained on drinking water containing 25 per cent D_2O, with ad libitum access to food and water. Every week for 3 consecutive weeks, 3 D_2O-fed mice and 3 controls were weighed and sacrificed. The livers were assayed gravimetrically for total lipid and colorimetrically for cholesterol,[10] and sections were taken for histologic examination and gravimetric determination of water content. Six more mice from each group were sacrificed, and individual liver homogenates were prepared for studies of hepatic lipogenesis from sodium acetate-2-C-14. Incubations were carried out for 4 hours under an atmosphere of 100 per cent oxygen in a buffer composed of K_2HPO_4 0.067 M, KH_2PO_4 0.042 M, $MgCl_2$ 0.006 M, nicotinamide 0.03 M, pH 7. The ratio of homogenate to buffer was 1:3. The reaction was stopped by the addition of 30 per cent metaphosphoric acid to pH 2. The suspension was saturated with KCl and then subjected to continuous ether extraction for 72 hours. The ether extract was reduced to dryness *in vacuo*, and the cholesterol and fatty acids were extracted from the dried residue with hot alcohol. Addition of digitonin precipitated the cholesterol, and extraction of the mother liquors with ether yielded the fatty acids. Cholesterol digitonide was assayed directly for radioactivity, and the cholesterol was also converted to the dibromide; this derivative, too, was assayed for radioactivity. The counts obtained by the two different methods were in agreement (±8 per cent). Fatty acids were treated with calcium acetate, and the calcium salts obtained by precipitation at $-20°$ C. The calcium salts were dissolved in acid, and the fatty acids purified by several reprecipitations. Fatty acids were prepared for counting by the method of Brady *et al.*[11] The radioactive assays were carried out with

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TABLE 1

BIOSYNTHESIS OF CHOLESTEROL AND FATTY ACID FROM ACETATE-1-C^{14} BY LIVER HOMOGENATES FROM MICE FED 25 PER CENT D_2O

Weeks on regimen	Group	Mouse No.	Cholesterol (cpm/mg. C × 10^3)	Fatty acid (cpm/mg. C × 10^3)
1	H_2O	1	1.65	12.64
		2	1.02	11.32
		3	1.75	5.66
		4	2.23	10.09
		5	1.27	8.39
		6	1.50	8.92
		Average	1.57 ± 0.16*	9.50 ± 0.99
	D_2O	1	0.75	4.05
		2	1.07	4.20
		3	1.79	7.16
		4	1.66	3.11
		5	1.65	6.95
		6	1.83	4.07
		Average	1.46 ± 0.18	4.92 ± 0.69
2	H_2O	1	1.62	1.21
		2	1.15	0.99
		3	1.19	0.30
		4	1.82	2.13
		5	1.81	0.81
		6	1.91	1.08
		Average	1.58 ± 0.14	3.26 ± 0.54
	D_2O	1	1.06	3.45
		2	1.05	3.28
		3	0.78	3.52
		4	1.59	3.94
		5	1.60	2.78
		6	0.65	2.93
		Average	1.12 ± 0.16	3.31 ± 0.17
3	H_2O	1	1.55	5.92
		2	0.96	5.11
		3	0.96	5.73
		4	0.43	5.81
		5	1.59	5.18
		Average	1.10 ± 0.21	5.55 ± 0.17
	D_2O	1	0.62	3.45
		2	0.79	3.28
		3	0.36	3.52
		4	0.68	3.94
		5	0.93	2.78
		6	0.80	2.93
		Average	0.69 ± 0.10	3.15 ± 0.22

* Standard error of the mean.

a gas-flow counter, the counts being made to a standard deviation error of ±0.3 per cent. The methods employed for the lipogenesis experiments are slight modifications of those previously described by Rabinowitz and Gurin.[12,13]

Results and Discussion

As D_2O feeding progressed, there appeared to be a progressive reduction in the cholesterogenic ability of liver, which fell from 90 to 70 to 60 per cent of that observed for the controls. The fatty acid synthesis in the D_2O-fed mice was roughly one half that of the controls in the first and third weeks. The lack of effect seen in the second week is inexplicable at the present time. The data are presented in TABLES 1 and 2.

TABLE 2

EFFECT OF D_2O-FEEDING (25 PER CENT) ON HEPATIC CHOLESTEROL AND FATTY ACID SYNTHESIS

Summary

Weeks	Diet	Cholesterogenesis		Fatty acid synthesis	
		Av. cpm/mg. C × 10^3	% Red'n.	Av. cpm/mg. C × 10^3	% Red'n.
1	H_2O	1.57 ± 0.16*		9.50 ± 0.99	
	D_2O	1.46 ± 0.18	8	4.92 ± 0.69	49
				$0.01 > P > 0.001$	
2	H_2O	1.58 ± 0.14		3.26 ± 0.54	
	D_2O	1.12 ± 0.16	30	3.31 ± 0.17	
		$P = 0.05$			
3	H_2O	1.10 ± 0.21		5.55 ± 0.17	
	D_2O	0.69 ± 0.10	38	3.15 ± 0.22	44
		$P = 0.10$		$P < 0.001$	

* Standard error of the mean.

TABLE 3

AUTOPSY DATA ON MICE FED 25 PER CENT D_2O

Week	Regimen	Average wt. (gm.)	Average liver wt. (gm.)	Per cent water in liver	Liver lipid (mg. %)	Liver cholesterol (mg. %)
1	H_2O	34.7	2.11	72.4	3185	284
	D_2O	31.3	2.11	72.0	3840	300
2	H_2O	28.7	1.74	72.3	3623	315
	D_2O	24.7	1.71	72.0	3645	248
3	H_2O	30.0	1.99	72.2	4778	527
	D_2O	28.7	2.19	73.0	4593	396

We turned our attention to the autopsy data in hopes of finding a clue that might explain the observed differences in lipogenic potential (TABLE 3).

It is evident that the major difference between the D_2O- and H_2O-fed mice is expressed in the liver weights as percentage body weight. Whereas the D_2O-fed mice were actually somewhat smaller in size, the livers of the two groups were almost the same size. The liver lipid and cholesterol levels were within the normal range for mice,[14,15] although cholesterol levels tended toward the low side. From these data one cannot ascribe the differences in lipogenesis to total liver lipid; lipid distribution, however, might be different in the two groups.

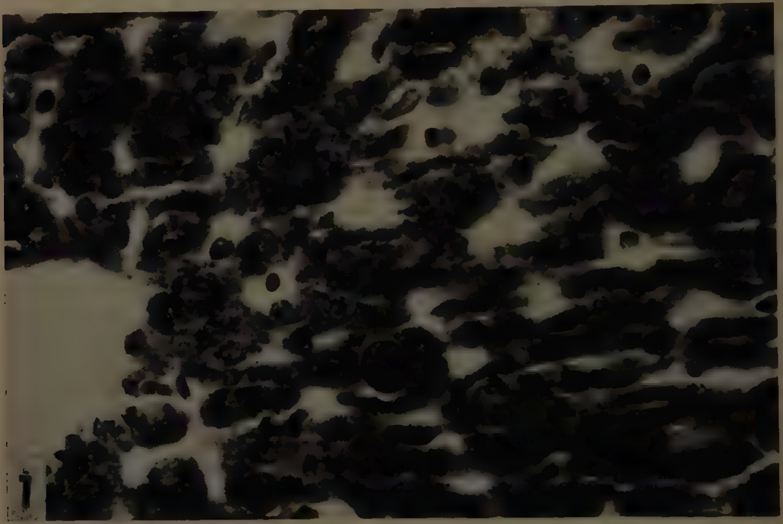

FIGURE 1. Liver—control. Large amount of basophilic material in the hepatic cells Toluidine blue. ×480.

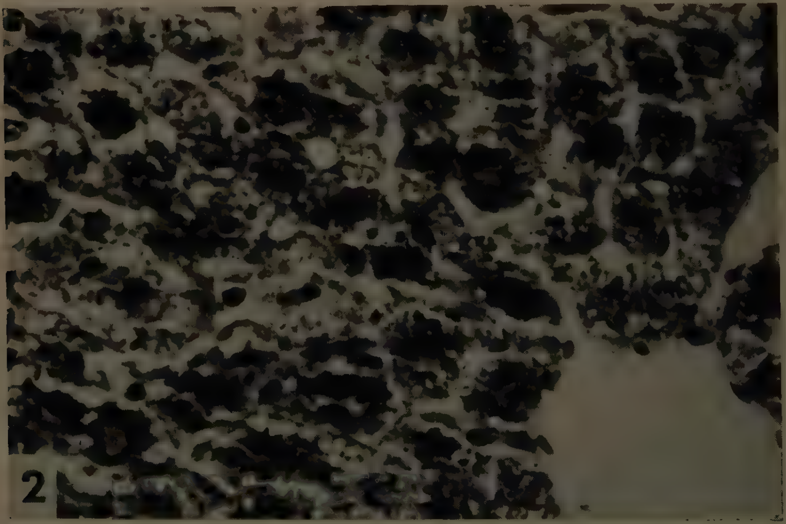

FIGURE 2. Liver—D_2O 25 per cent fed for 3 weeks. The cells are swollen, and the basophilic material has almost disappeared from the cytoplasm; only perinuclear clumps are visible. Toluidine blue. ×480.

Histologic examination revealed that the main changes in the livers of the D_2O-fed mice appeared to involve the distribution of ribose nucleic acid (RNA) and its concentration within the cytoplasm, and a moderate increase in sudanophilic material (both alcohol-soluble and alcohol-insoluble) in the cytoplasm. These changes are visible by the seventh day of treatment and increase during the second and third week (FIGURES 1 and 2).

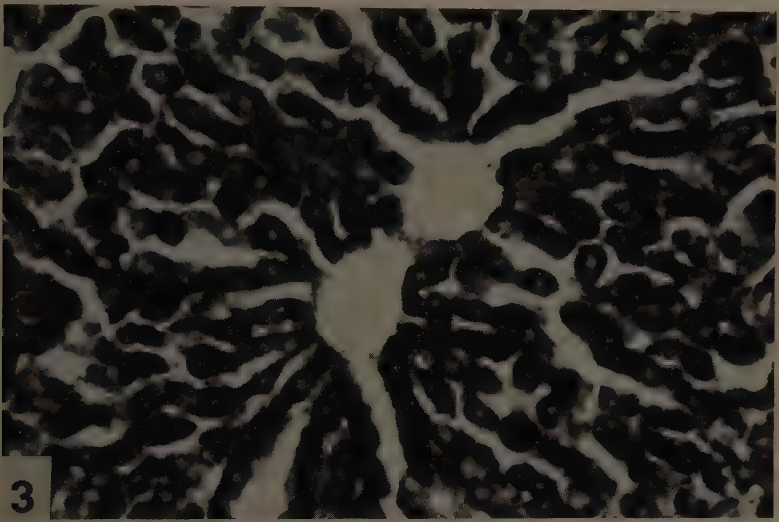

FIGURE 3. Liver—control; frozen section. Abundant sudanophilic material uniformly distributed in the cytoplasm of the hepatic cells. Sudan black. ×190.

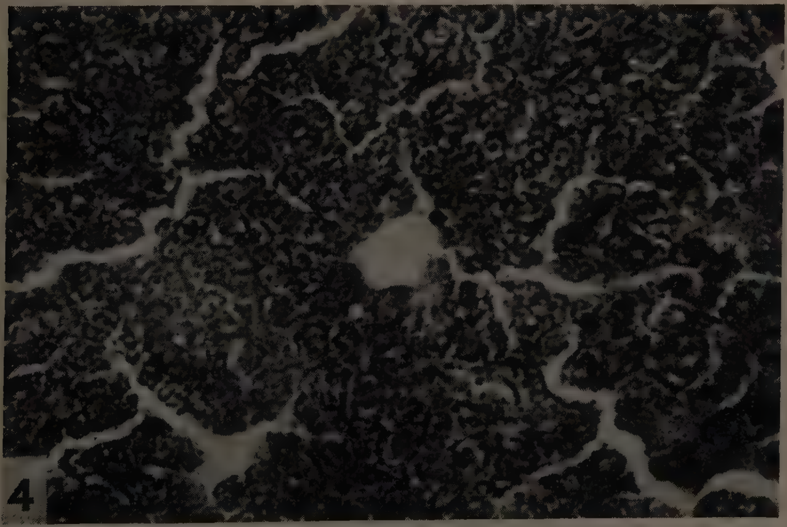

FIGURE 4. Liver—D_2O 25 per cent fed for 3 weeks. The sudanophilic material is distributed in large clumps in the cytoplasm. Sudan black. ×190.

On the seventh day, the cytoplasm appears less uniform than the control, with increased vacuolization and large aggregates of basophilic material. The aggregation of basophilic material (RNA) within the cytoplasm continues progressively, the aggregates appearing either at the cellular or the nuclear membrane. The most dramatic changes appear in the second week. The hepatic cells appear edematous and swollen, with the cellular border clearly

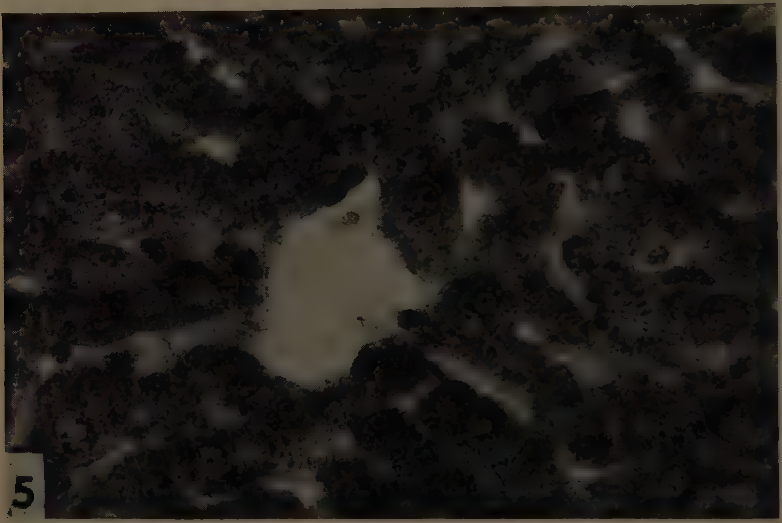

FIGURE 5. Liver—control; paraffin section. The amount of sudanophilic material is decreased as compared to FIGURE 3 after treatment with alcohol and xylol.

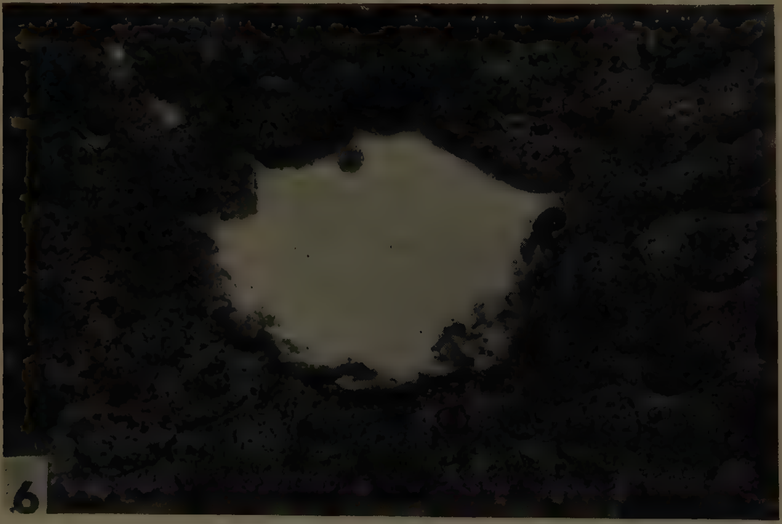

FIGURE 6. Liver—D_2O 25 per cent fed for 3 weeks; paraffin section. Larger amount of sudanophilic material is left after alcohol and xylol treatment. Sudan black. ×480.

distinguishable; very little basophilic material is left in the cytoplasm, which has become completely vacuolized. In these livers there is an increase in sudanophilic material, which is present in a granular distribution (FIGURES 3 and 4). The sudanophilic material in the livers of the D_2O-fed mice was more resistant to the usual lipid solvents than was that in the normal livers (FIGURES 5 and 6).

TABLE 4
EFFECT OF D_2O SOLUTION ON BIOSYNTHESIS OF CHOLESTEROL AND FATTY ACIDS BY MOUSE LIVER HOMOGENATES

Expt. No.	Solution	Mouse No.	Cholesterol (cpm/mg. C × 10^4)	Fatty acid (cpm/mg. C × 10^4)
1	H_2O	1	0.89	2.77
		2	1.03	2.44
		3	1.15	2.99
		4	0.98	2.84
		5	1.07	2.99
		6	0.89	3.02
		Average	1.00 ± 0.04*	2.84 ± 0.09
	D_2O	1	1.45	3.15
		2	1.18	3.38
		3	1.40	3.26
		4	1.30	3.05
		5	1.38	3.29
		6	1.16	3.33
		Average	1.31 ± 0.05	3.24 ± 0.05
2	H_2O	1	1.42	2.64
		2	1.26	2.85
		3	1.40	2.75
		4	1.16	2.55
		5	1.30	2.61
		6	1.20	1.88
		Average	1.29 ± 0.04	2.55 ± 0.14
	D_2O	1	2.08	3.75
		2	2.34	3.47
		3	2.15	3.69
		4	2.41	3.86
		5	2.01	3.98
		6	1.74	3.37
		Average	2.12 ± 0.10	3.69 ± 0.09

* Standard error of the mean.

The lesions progress uniformly throughout the liver and show no preferential localization. These findings suggest that D_2O, at the concentration administered, produces pronounced structural changes in the cytoplasm. Further studies have suggested a decrease in the number of round mitochondria, with swelling and rearrangement of the filamentous mitochondria.

These findings suggest two possible causes for the decreased lipogenesis displayed by the deuterated livers: (1) diffusion of lipid within the liver, or (2) interference with basic structural or enzyme elements.

To test the actual effect of D_2O on the lipogenic system, two series of incubation experiments were carried out: one in which the buffer was dissolved in D_2O ($>$99.5 per cent), and another using the normal aqueous system. Solutions of buffer were taken to dryness *in vacuo* and reconstituted in H_2O or D_2O as required. The results of these experiments are detailed in TABLES 4 and 5.

In these experiments we see that the presence of D_2O in the medium resulted in a significant enhancement of both cholesterol and fatty acid synthesis. The effect of D_2O on enzyme reactions is variable, as a review of the early literature has shown.[16] A study of the activities of various enzymes in the livers of D_2O-fed rats has shown reductions in some (catalase, diphosphopyridine nucleotide, cytochrome reductase, cytochrome oxidase), enhancement in some (uricase, arginase), and questionable changes in succinic dehydrogenase.[6]

TABLE 5

EFFECT OF D_2O (75 PER CENT) ON HEPATIC LIPOGENESIS IN MICE

Summary

Expt. No.	Medium	Cholesterogenesis		Fatty acid synthesis	
		Av. cpm/mg. C × 10^4	% Increase	Av. cpm/mg. C × 10^4	% Increase
1	H_2O	1.00 ± 0.43*		2.84 ± 0.09	
	D_2O	1.31 ± 0.05	31	3.24 ± 0.05	14
		($P < 0.001$)		($0.01 > P > 0.001$)	
2	H_2O	1.29 ± 0.04		2.55 ± 0.14	
	D_2O	2.12 ± 0.10	65	3.69 ± 0.09	45
		($P < 0.001$)		($P < 0.001$)	

* Standard error of the mean.

There appears to be enhancement of the activity of the enzymes involved in lipid synthesis.

The reduced lipogenesis seen in the mice fed D_2O would seem to be due to the derangement of various structural elements of the cells, rather than to inhibition of enzyme activity per se. The increased lipid observed histologically may be due to enhanced lipogenesis during the first days of the feeding period.

Summary

Swiss mice were maintained on a regimen of 25 per cent D_2O for three weeks. The mice were slightly smaller than H_2O-fed controls, but the liver weight body weight ratio was greater. There were no significant differences in liver lipid or cholesterol. Histologic examination showed progressive vacuolization and loss of basophilia, with changes in the mitochondrial distribution in the cytoplasm. These alterations did not show any specific localization in the hepatic lobule.

There was a progressive reduction in the ability of liver homogenates from D_2O-fed mice to convert acetate-2-C-14 to cholesterol and fatty acid. Incu-

bation of normal mouse livers in media containing 75 per cent D_2O resulted in significant enhancement of cholesterol and fatty acid biosynthetic capacity. This reduced lipogenesis in D_2O-fed mice appears to be due to derangements in cell structure, rather than to inhibition of enzyme activity.

Acknowledgments

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PHYSIOLOGICAL EFFECTS OF D_2O IN MAMMALS*

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Within a few months after the announcement of the discovery of deuterium, a large number of investigators began to study the biological effects of heavy water. However, because of the scarcity and high cost of D_2O, relatively little work was carried out on mammals. The most extensive systematic investigation of the pharmacology and toxicology of D_2O in mice was reported by Barbour and his co-workers, beginning in 1935; the significant findings were summarized by Barbour in 1937.[1] Although limited by the supply of heavy water, Barbour and his co-workers made a number of important observations. They established, among other things, that mice could not survive replacement of more than about one third of their body water by D_2O; that the metabolic rate was increased in the early stages of D_2O intoxication; and that the animals showed evidence of autonomic imbalance, that is, that D_2O apparently had a sympathomimetic action.

In recent years the cost of D_2O has dropped markedly, and large quantities of heavy water are now available at a price low enough to permit the study of ts effects on animals larger than mice. This communication is a summary of iur observations made during the past four years of the effects of D_2O on rats.

Methods

In the majority of experiments, Sprague-Dawley female rats were used. Their ages ranged from 3 to 8 months, and their weights varied between 190 and 260 gm. In some of the earlier experiments, the rats received 100 per cent D_2O as drinking water for a few days before being given 50 per cent D_2O; in more recent work, they have been given 50 per cent D_2O in tap water throughout the experiment. Their food consisted of Rockland rat diet ad libitum.

Most of the specialized techniques used in the studies reported here have been described in other communications.[2-6]

Results

Little change was observed during the first few days that the rats were drinking heavy water. There was initially a slight weight loss, or at least a failure to gain at the same rate as the controls, although this loss may have been attributable to the fact that the environment of the rats had been disturbed,[7] and not to any specific action of D_2O. After about a week or 10 days, depending on the size of the rat, when 15 per cent of the body water had been replaced by D_2O, the animals began to show signs of hyperactivity, and at 20 to 25 per cent replacement they became very hyperexcitable, often convulsing when stimulated. At the same time, they stopped grooming themselves; possibly for this reason, ulcers appeared on their paws, and their tails became necrotic. At 30 per cent replacement, the animals became comatose and refused to eat;

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* The work described in this article was performed under the auspices of the United States Atomic Energy Commission, Washington, D.C.

their weight dropped, sometimes precipitously; and they died apparently from respiratory depression, a few with terminal convulsions.

We have not carried out a systematic histologic study of the tissues of D_2O-treated rats. Grossly, an increase in the size of the liver and of the adrenal glands was observed.[2] The increase in the weight of the liver, as per cent of total body weight, reached 50 per cent in some cases, whereas a 60 per cent increase in relative adrenal weight was observed. The data in TABLE 1 show that this increase in liver weight was a true hyperplasia, since the concentration of deoxyribonucleic acid phosphorus per unit weight liver was decreased by an insignificant amount, while the total weight of the liver increased nearly 40 per cent.

Analyses of blood or plasma of rats drinking D_2O were carried out in the course of deuteration. FIGURES 1 and 2 show some of the results that were obtained. It is clear that there was a steady increase in nonprotein nitrogen and urea, and a decrease in glucose and plasma protein. There were also increases in blood lactic acid and inorganic phosphate (not shown in FIGURES 1 and 2).

TABLE 1

EFFECT OF D_2O ON DNA CONTENT OF RAT LIVER

	Average with standard deviation	
	Control	D_2O-treated
No. rats	4	6
Days on D_2O	—	26
Plasma D_2O, mole %	—	28.8 ± 1.4
DNA-P, mg./100 gm.	28.7 ± 2.2	25.0 ± 4.4
Liver weight, % of body weight	2.26 ± 0.16	3.12 ± 0.32
Adrenal weight, % of body weight	0.022 ± 0.002	0.032 ± 0.003

These changes qualitatively resemble those seen in adrenalectomized rats.[8] Since we were unable to study the effect of D_2O directly on adrenalectomized animals because of the necessity of supplying continuous hormone therapy, we used hypophysectomized rats of the same strain and sex. In these animals the adrenals atrophy rapidly after the pituitary is removed. It can be seen from FIGURE 3, in comparison with FIGURE 2, that the changes observed in unoperated animals developed much more rapidly in the hypophysectomized rats, 3 of the 4 animals dying with plasma D_2O levels of less than 20 mole per cent. The fourth rat in the series was somewhat more resistant, and died on the 15th day, with about 25 per cent replacement.

In this connection it may be noted that adding 0.5 per cent NaCl to the drinking water of unoperated rats decreased their tolerance to D_2O, the survival time on 50 per cent D_2O dropping from 31 to 25 days.

The effect of D_2O on the erythrocyte count in rats is shown in FIGURE 4. It is evident that the animals became markedly anemic, the red cell count dropping to 40 per cent of the normal level; in some rats, the number of erythrocytes was reduced to less than 20 per cent of normal. When the animals were then given H_2O to drink, the erythrocyte count returned slowly to normal. A consistent picture was not obtained with the white cell count. Both the lymphocyte and granulocyte counts decreased, the former generally more than the latter; however, the change was not as dramatic as that seen with the erythrocytes. In mice, on the other hand, Gustavson and Häggqvist[9] observed a 60 per cent decrease in white count, and only a 15 per cent decrease in numbers of erythrocytes. Their mice drank 50 per cent D_2O for only 14 to 16 days, and with further ingestion the anemia might have become more severe.

The increase in blood urea nitrogen could be due either to an increased production or to a decreased excretion. The former hypothesis was tested in nephrectomized rats, according to the technique of Engel.[10] It is clear from TABLE 2 that there was about a 20 per cent increase in rate of urea production, probably not enough to account for an increase of 170 per cent in blood urea levels if the kidneys were functioning properly. It is probable that the increased ureagenesis merely reflects the increase in liver size previously noted.

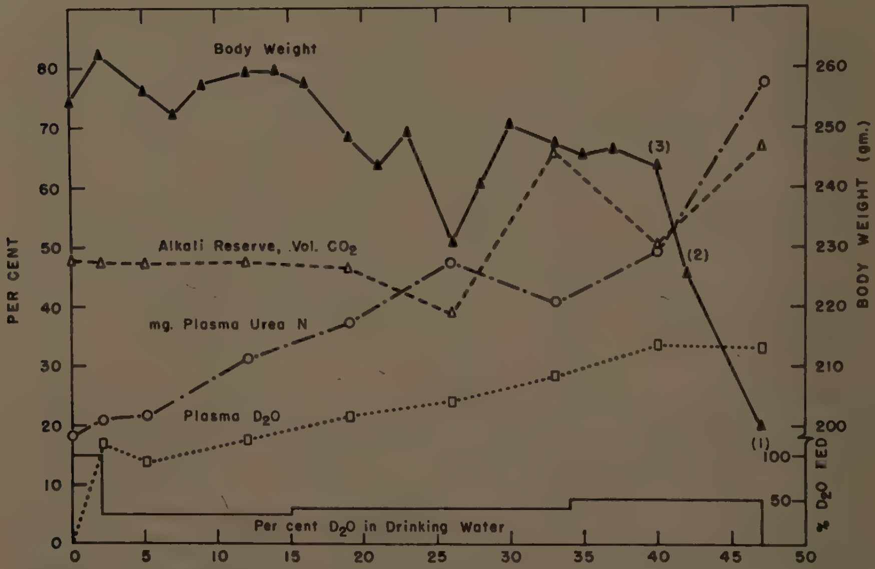

FIGURE 1. Effect of D_2O on body weight, alkali reserve, and plasma urea in rats. Numbers in parentheses indicate number of survivors.

Studies of renal function were then carried out. Since the details of this study have been presented elsewhere,[3] it is sufficient to note only that both glomerular filtration (measured by creatinine clearance) and renal plasma flow (measured by *p*-aminohippurate [PAH] clearance) were decreased by over 60 per cent. However, within 5 days after restoring H_2O to the animals, both clearances returned to normal. These data suggested that the effect of D_2O on renal function was indirect, since the reduction took place in the absence of any consistent morphologic or biochemical change. This view is substantiated by the observation that the capacity of kidney slices to accumulate PAH *in vitro* was the same for both normal and D_2O-treated rats.[4] However, high concentrations (60 per cent) of D_2O *in vitro* markedly decreased the capacity of slices to accumulate PAH, with little effect on oxygen consumption.

The decrease in blood glucose suggested that a study of the effect of injection of glucose might be profitable. FIGURE 5 shows that 30 min. after intraperitoneal injection of 1 gm./kg. of glucose the blood levels in D_2O-treated rats were almost 50 per cent higher than those in normal rats. However, within 5 hours after injection, the levels in the deuterated animals had fallen nearly to the original value, about 25 per cent below normal.

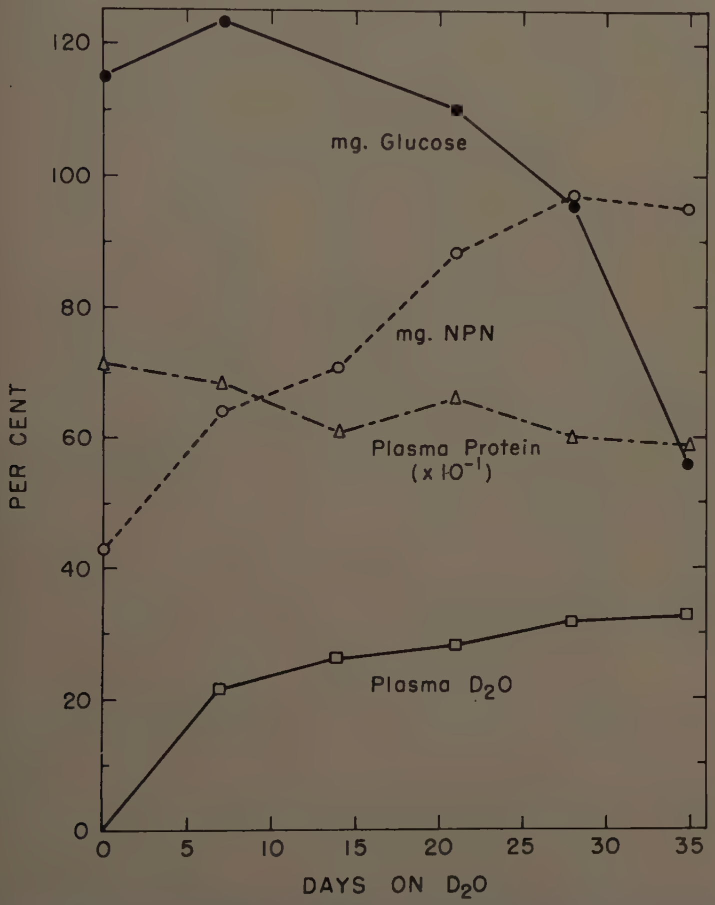

FIGURE 2. Effect of D_2O on blood glucose and NPN and on plasma protein in rats. Four rats were used at each point except the last one, which represents only one animal.

Two days later the rats were killed, and the data presented in TABLE 3 were obtained. There was a further decrease in blood glucose, and an almost total lack of liver glycogen. TABLE 3 also indicates the magnitude of the changes in body weight and plasma urea effected by D_2O in this particular series of rats.

A number of enzyme assays have been carried out in tissues of rats drinking D_2O. Most of these results have been reported elsewhere.[2,5] Few of the changes were striking; a 35 per cent decrease in the activity of DPN-cytochrome *c* reductase in the livers of deuterated rats was probably the most impressive change. The catalase activity of the liver was also decreased by approximately the same amount, but this change is probably of little consequence for the survival of the animal. Increases were noted for arginase, uricase, alanine transaminase, and tryptophan pyrrolase. However, the response of the deuterated rat to injection of tryptophan was markedly suppressed; the activity of tryptophan pyrrolase in the tryptophan-adapted rat drinking D_2O was only one third that seen in the normal adapted animal.

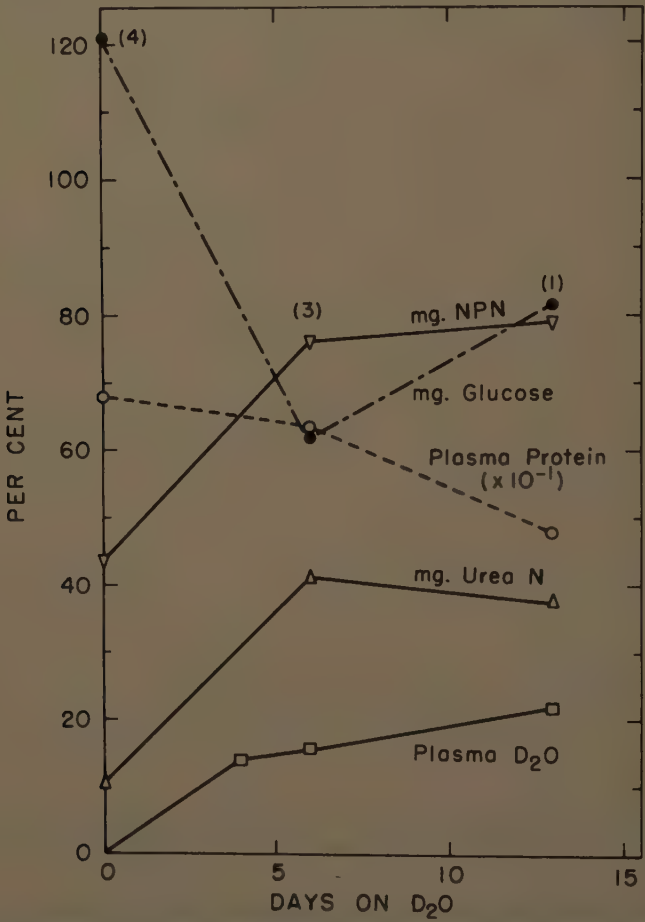

FIGURE 3. Effect of D_2O on blood glucose, NPN, and urea, and plasma protein in hypophysectomized rats. Numbers in parentheses indicate the number of survivors.

The capacity of liver and kidney mitochondria to esterify inorganic phosphate was not markedly altered by deuteration of the animal. No effects were observed on either oxygen consumption or phosphate esterification when succinate was used as a substrate. With malate or α-ketoglutarate, both oxygen consumption and phosphate esterification were diminished, but the efficiency of the process, as evidenced by the P:O ratio, was unchanged.[5]

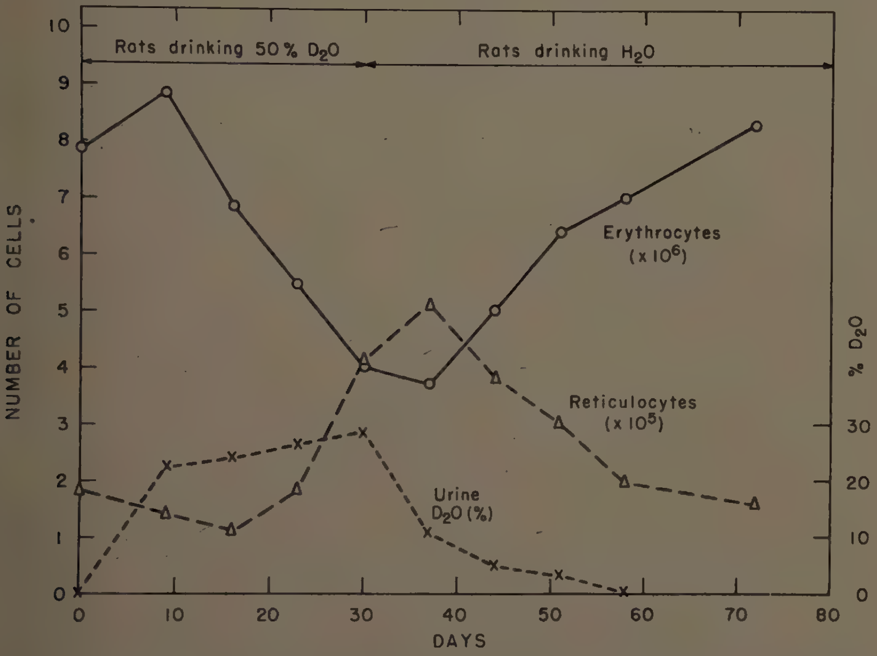

FIGURE 4. Effect of D_2O on erythrocyte and reticulocyte counts in rats. Averages of 6 rats.

TABLE 2
UREAGENESIS IN D_2O-TREATED RATS

	Average* with standard deviation	
	Control	D_2O-treated
Initial blood urea, mg. %	9.7 ± 0.5	26.5 ± 0.3
Rate of increase, mg./hr./100 gm. body weight	3.23 ± 0.05	3.88 ± 0.10

* Eight rats per group.

Experiments in which tissues from normal animals were assayed in the presence of 30 per cent D_2O have shown that in most cases the solvent effects of D_2O were rather slight. A notable exception was fumarase, for which D_2O is both a solvent and a substrate. The forward reaction, the hydration of fumarate, was inhibited about 20 per cent in the presence of 33 per cent D_2O, while the reverse reaction was inhibited about 10 per cent (in preparation).

Little work has been done with deuterated substrates. Our experience with completely deuterated succinic acid has shown that it was dehydrogenated enzymatically at only one fifth the rate of ordinary succinate. Succinate containing 30 atom per cent deuterium, the maximum deuterium content to be expected under physiological conditions, was dehydrogenated at 80 per cent of the rate at which the normal compound was metabolized.[6]

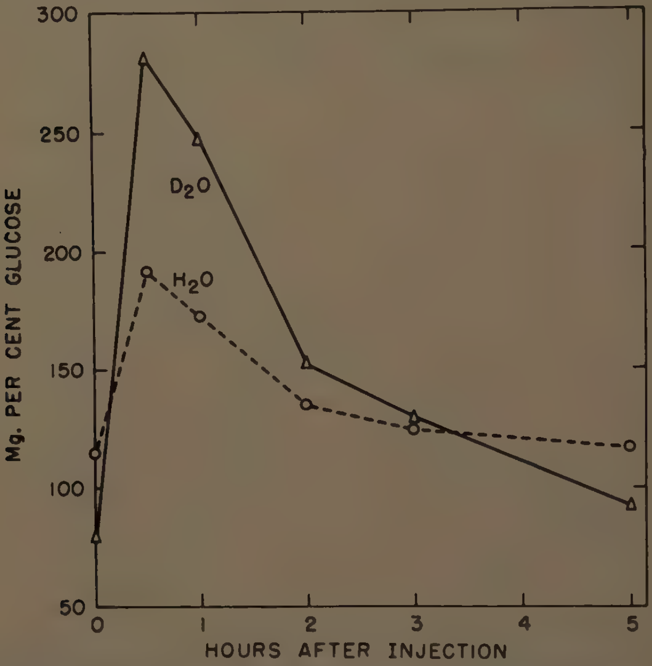

FIGURE 5. Effect of D_2O on response of blood sugar to injection of glucose. Averages of 5 rats in each group.

TABLE 3

BLOOD GLUCOSE, LIVER GLYCOGEN, AND PLASMA UREA IN D_2O-TREATED RATS

	Average with standard deviation	
	Control	D_2O-treated
No. rats	6	4
Days on D_2O	—	29
Plasma D_2O, mole %	—	28.7 ± 0.5
Blood glucose, mg. %	105 ± 4	50 ± 6
Liver glycogen, %	3.90 ± 0.60	0.04 ± 0.04
Plasma urea, mg. %	18 ± 4	65 ± 6
Weight change, gm.	+41 ± 10	−36 ± 5

Discussion

Many of the data presented above indicate a disturbance of adrenal function: the increase in size of the adrenals; the terminal blood picture, with elevated

NPN and decreased glucose levels; and the impairment of renal function. It would be erroneous, however, to consider that the animals were functionally adrenalectomized. The activities of transaminase and arginase in rat liver are known to decrease after adrenalectomy,[11,12] and the fact that these activities did not decrease in animals drinking D_2O makes untenable the concept that the deuterated rat behaves like an adrenalectomized rat. Thus the production of adrenal hormones does not seem to be inhibited. It is more probable either that the metabolism of the deuterated rat is so altered by D_2O that additional amounts of adrenal hormones are of no benefit, or that the animal may have a higher requirement for adrenal hormones than can be met even by a hypertrophic adrenal cortex. It seems certain that some adaptation process, which is influenced by the adrenals, takes place in rats drinking D_2O. The decreased survival of hypophysectomized rats and the increase in the basal level of tryptophan pyrrolase are consistent with this idea.

It is possible that the principal defect in these animals is in the utilization of carbohydrate. The decrease in blood sugar and the abnormal response to injected glucose suggest an impairment of the mechanisms controlling the glucose level in the blood. There seems to be an extensive breakdown of protein, but gluconeogenesis apparently does not occur at a sufficiently rapid rate to maintain the blood sugar level within the normal range.

Another aspect of the toxicology of D_2O to be considered is its pronounced effect on rapidly growing tissues. As indicated in other papers presented at this conference, D_2O interferes with the growth of cancer cells and reproductive and embryonic tissues (elsewhere in these pages), as well as hematopoietic tissues. Probably there is an inhibition of synthesis of one or more compounds necessary for cell division. However, this process may not be entirely unselective, since we observed less of an effect on leukocyte production than on erythrocyte production.

The cause of death of deuterated rats is not entirely clear. The animals suffer from anemia, renal damage, malnutrition, central nervous system damage, possibly adrenal insufficiency, and very likely many other disturbances, any of which could contribute to the lethal process.

To attempt to relate the lethal effects of D_2O to a specific effect at the molecular level seems fruitless at the present time. In enzymatic reactions, at least four aspects must be considered: (1) solvent effects of D_2O, (2) primary isotope effects, (3) secondary isotope effects, and (4) the effects of deuteration of the enzyme. For *in vivo* studies, a fifth factor, the effect of deuteration on the concentration of the enzyme as well as on its activity, has to be taken into account. It seems probable that almost every reaction in the body will be modified at least to some extent, so that although no one reaction would appear so be significantly altered, there is an over-all disturbance in metabolic relationships that leads to dysfunction and death of the animal.

Summary

(1) Rats drinking D_2O died when about one third of their body water was replaced by D_2O. Hypophysectomized animals were considerably more sensitive to D_2O than were normal rats.

(2) Evidence was obtained for impairment of kidney function, anemia,

disturbed carbohydrate metabolism, central nervous system disturbances, and altered adrenal function; no specific cause of death can be definitely stated at the present time.

(3) Studies on the effects of D_2O on mammalian enzymes *in vivo* and *in vitro* have indicated a number of changes, few of which by themselves seem to be of much consequence. It seems probable that the toxic action of D_2O is attributable to the summation of many small changes in rates of enzymatic reactions in the body.

Acknowledgments

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THE INHIBITION OF MITOSIS BY DEUTERIUM*

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In a recent issue of these *Annals*, we described in detail a number of investigations concerning the antimitotic action of heavy water.[1] It was observed that, depending upon the conditions of deuteration, cell division in eggs of the sea urchin *Arbacia punctulata* could be delayed or arrested reversibly. The present report, based in part upon those experimental results, will contain only summary descriptions of our observations and will be concerned, instead, primarily with mechanisms. To this end we shall first call attention to the universality of the antimitotic action of D_2O, then proceed to outline the several types of hypotheses that have been advanced to account for this effect and, finally, offer a selection, based upon our data, from among those proposals. For fuller discussion of the various experimental techniques and a more complete review of the pertinent literature, the reader is referred to the earlier paper.[1]

It has become evident during the contemporary renaissance of interest in the biology of deuterium that one of the most universal responses of organisms to excess environmental deuterium is delay, pathology, or cessation of cell division. Descriptions of the antimitotic action of D_2O offered by earlier workers, for example those of Lucké and Harvey,[2] Ussing,[3] and Cagianut,[4] have been extended to a variety of microorganisms, invertebrates, and mammals through the efforts of contemporary students of the problem, notably Katz and his co-workers,[5] and the Berkeley group.[6] Certainly the effects of deuteration in biological systems are varied and complex, as the reports in this monograph reveal, but directed scrutiny of these reports reveals clearly that interference with cell division is one effect that transcends phylogeny: thus organisms as remote from one another as algae, echinoderms, and mammals show it as a primary phenomenon. Cell division is indeed complicated, even considered in relation to other biological processes, but it is not as complicated as the survival of a mammal under stress, nor is it as complicated as the growth of a mammalian tumor or the growth and metabolism of a population of microorganisms. To put it in other terms, we understand rather more about the indispensable steps in cell division (Mazia[7]) and about their time sequences and energetic requirements (Swann[8] and Gelfant[9]) than we do about such general attributes of living systems as growth, adaptation, and survival. It is hence appropriate to focus attention upon the antimitotic effect of D_2O, since at least in such systems as the sea urchin egg, cell division can be isolated ex-

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perimentally from growth, adaptation, and survival. This cell is poised for a long series of successive mitoses following the triggering event of fertilization; here the mitoses do proceed in the absence of growth and probably with considerable independence of the cytoplasmic volume, as has recently been shown by Rustad and Rustad.[10]

We undertake now to restate very briefly the five major types of proposals that have been made to account for the biological effects of deuterium enrichment, and particularly for their interference with cell division.

(1) According to the first of these proposals the many catenary and coupled chemical reactions of metabolism make up a complex steady-state process, and the replacement of hydrogen by deuterium changes the rates of a large proportion of the component transformations. This results usually in a retardation of the metabolic machinery at numerous critical points. What follows may be not merely a generalized depression of metabolism, but also a qualitative alteration of metabolic pathways, since the maintenance of the normal pattern depends upon the steady delivery of critical metabolites to certain compartments of the living cell. Thus, for example, if the entire electron-transport system suffered a sharp rate loss upon deuteration, the cell might soon "see" smaller deliveries of ATP from the mitochondria to the soluble phase. Quite possibly, however, the concentration of ATP in the soluble phase serves as a signal by which the rate of amino acid activation therein is controlled. Deuteration could then serve indirectly to switch the activity of soluble enzyme systems from, let us say, protein synthesis to, for example, purine degradation. Thus isotopic substitution might not only depress the over-all intensity of metabolic transformations but, by the production of false signals such as those described, could alter qualitatively the cell's normal responses, whether they are chemical, mechanical, or electrical, to messages arriving from outside.

(2) This proposal derives directly from the first, but specifies more exactly the nature of the isotope effect and acquires thereby a certain predictive power. Let us suppose that a cell, perhaps a microorganism, grows normally in a medium containing the nutrient sources A, B, and C. Growth can be supported by the molecular species A alone, but optimal growth occurs only when all three substances are present. We may imagine that from A, B, and C the cell ultimately obtains metabolites X, Y, and Z, and that these are indispensable intermediates required for the synthesis of materials used in growth and/or cell division. The simple linear scheme shows that the cell can manage

$$\begin{tikzcd}
A \arrow[r, "k_1"] & D \arrow[r] & G \arrow[r] \arrow[d] & \cdots \arrow[r] & X \arrow[dr] & \\
B \arrow[r] & E \arrow[r] & H \arrow[r] \arrow[d, shift right] & \cdots \arrow[r] & Y \arrow[r] & S \\
C \arrow[r] & F \arrow[r] & I \arrow[r] \arrow[u, shift right] & \cdots \arrow[r] & Z \arrow[ur] &
\end{tikzcd}$$

with A alone since, if there is a sufficient environmental supply, its product G can be converted to H, making B unnecessary. Likewise, if sufficient H is present, its conversion to I will circumvent the requirement for C. Nevertheless an environmental supply of B and C will permit more rapid accumulation of the critical intermediates X, Y, and Z, because the diversion of G and H to alternate pathways will not be required. Suppose now that the reaction $A \rightharpoonup D$, whose rate constant is k_1, requires the rupture of a bond to hydrogen, and hence has a large primary kinetic isotope effect. Upon deuteration, growth would be very much slowed in the complete medium $A + B + C$, and might cease altogether in the minimal medium A because the rate of production of the critical intermediates, X, Y, and Z, would fall below that required to maintain the steady-state synthetic pathway S into which they normally enter. If the only step showing a very large isotope effect were $A \rightharpoonup D$, it would be possible, in a complete medium, to reverse the growth inhibition due to deuterium by substituting any of the intermediates in the reaction chain $D \rightharpoonup G \rightharpoonup \cdots \rightharpoonup X$. In the minimal medium A, the inhibition would be reversible only upon supplementation with D or G. This type of hypothesis therefore leads to the prediction that cells failing to grow or divide in a deuterated medium containing the ordinary nutrients should grow and divide if the proper new supplements are added.

(3) These proposals deal more directly with cell division. They concern the mobilization of monomeric precursors for macromolecules that must be multiplied before the microscopically detectable events of mitosis begin. DNA is an obvious candidate, since it must be doubled prior to any normal mitosis. Quite possibly one or more of the cell's RNA compartments must also be doubled. Furthermore, the cell must synthesize, prior to each mitosis, the proteins and nucleoproteins that are assembled into the mitotic machinery, and their constituent purines, pyrimidines, and amino acids would likewise have to be accumulated. Following the accumulation of the necessary concentrations of monomer, the macromolecules themselves would have to be assembled. The proposal is that deuteration blocks one or more steps leading to the synthesis of the precursors or, alternatively, that it inhibits the polymerization reactions. This hypothesis too is a variant of the first proposal.

(4) We assume in this case that the metabolism of the deuterated cell, albeit slowed and distorted, ultimately suffices to mobilize the precursors described in the third proposal. We assume further that the *rate* of appearance of the polymers will not be influenced greatly. Confining our statement to nucleic acid, we specify now that one or another pathology enters the condensation process or the behavior of the completed macromolecules without any serious change in the rate of their completion. First, it is possible that deuteration can increase the *error rate* in the polymerization process without changing drastically the over-all assembly time. Such errors in the sequence of nucleotides, if of sufficient magnitude, would produce mutations. Furthermore, these polymers owe their "native" configurations to multiple hydrogen bonding of an *intramolecular* kind, and the substitution of D for H in these bonds might be expected to produce an "instability" in the polymer molecules. The "instability" is not yet defined; approximately, it is supposed to be a biological instability that, in a mild form, would manifest itself again as mutation. In a severe form, however, it is envisaged that the entire mitotic process could be disorganized because, for example, the component polynucleotide chains of

DNA would either not "stick" together helically or would "stick" too tightly, depending upon the direction of the isotope effect upon H-bond strength. The failure of normal geometry in DNA might well disrupt such microscopic features of mitosis as chromosomal condensation, spiralization, and splitting, leading to severe mitotic aberrations and to death of the cell. That deuteration of internally H-bonded macromolecules can, in fact, lead to significant changes in stability and in configurational properties has been shown by Calvin *et al.*,[11] and the case of H-bond cross-linked systems is being investigated by us.

(5) In the fifth proposal, we consider that although deuteration may affect the dividing cell in all of the ways suggested above, the critical point of interference is none of those thus far listed. It is recalled, instead, that in a normal mitosis and in the subsequent cytokinesis in animal cells very drastic rearrangements are made in the structure of the ground cytoplasm. These rearrangements are fundamentally condensations of macromolecules and particulates to form ramifying aggregates, which are locally ordered to produce the microscopically observable machinery of cell division, that is, the spindle, the asters, the cell centers (insofar as these change from interphase to prophase), and the organized regions of the cell cortex that form the cleavage furrows. It is assumed that the ground substance of the cytoplasm, a complex, polyphasic colloidal system, is already somewhat structured by virtue of H-bond and salt bridge contacts among its constituent macromolecules, even in the "resting" state.[12] The locally ordered gelation processes that assemble the mitotic machinery must also, to a greater or lesser extent, depend upon hydrogen bond cross-linking. The proposal is that deuterium acts directly upon all of these multiple hydrogen-bonded systems, in the resting cytoplasm as well as in the dividing cell, in such a way that the local structure changes that would normally occur during mitosis are blocked. This can happen in two ways, depending upon the direction again of the isotope effect on H-bond "strength." If D-bonds and D-bond cross-links are weaker than H-bonds in the same system, the blockade would result from a kind of melting. If the D-bonds are stronger, the blockade would arise from a "freezing" process. Thus this fifth hypothesis regards the critical site of the antimitotic action of deuterium to be the mitotic apparatus and its accessory structures, either during assembly or in operation.

The list given above is not meant to be an exhaustive survey of the possible modes of action of deuterium on cells. Rather, the list as assembled is meant to represent the general classes of effect whose operations have been invoked in the literature or in discussions.

We turn now to a summary of our own observations on the inhibition of cell division in sea urchin eggs by deuterium oxide:

Depending upon the concentration of D_2O in the medium, the cleavage of fertilized sea urchin eggs is either delayed or totally blocked. When the deuterium content of the medium is below 30 atom per cent, the time from fertilization to the first division is a rapidly increasing function of the deuterium concentration. There is no significant alteration in the kinetics of cleavage for the population so that, for example, all of the cells in a control suspension might divide between 50 and 55 min. after fertilization whereas, in the experimental suspension, with 25 per cent D_2O, all of the cells might typically divide in the interval between 63 and 68 min. The absolute length of the delay period increases rapidly with decreasing temperature for a given concentration of D. As the environmental D_2O content is raised from 30 to 50 per cent, the delay becomes a more rapidly increasing function of the D concentration and the cleavage kinetics change from the control, so that the interval between the divisions of the first and last cells capable of cleaving becomes very large. When the concentration of D_2O is raised beyond 70 per cent, cell division is totally arrested in this egg.

The effects described above are entirely reversible. Cells that have remained blocked in D_2O for intervals that are long relative to the normal viability period of the egg, begin to divide or to prepare for division as soon as the D_2O is washed out and replaced by normal water.

The antimitotic effect is obtained not only in the first cleavage cycle, but in any division cycle during which the cells are immersed in D_2O.

At least some replication processes continue during the time that cells are in D_2O, for if their residence therein has been longer than the period of one normal division, the blocked cells undergo simultaneous multiple cleavages when returned to H_2O. These multipolar mitoses suggest very strongly that whereas the mitotic spindles and cleavage furrows have remained unchanged during deuterium treatment, the cell centers have been able to divide on schedule. It is possible that the DNA also replicated during the mitotic blockade, although an unequivocal demonstration has not yet been given. The question is under study at the moment, with an attempt being made to follow the incorporation of tritiated thymidine into normal and deuterium-blocked fertilized eggs.

The antimitotic effects of D_2O on the sea urchin egg are virtually instantaneous, as is shown by the results of the stage-sensitivity experiments to be described below. Breakage of the mitotic block upon reimmersion of the eggs in H_2O requires no more time than its imposition. Therefore, the rate of equilibration of external D_2O with intracellular water must be very high, and the exchange rate between H and D at the sites critical for the antimitotic effect must likewise be very high. This suggests that the sites are probably on amino, carboxyl, and hydroxyl groups, and on other organic functions with freely-exchangeable hydrogens. The high speed of equilibration between external D_2O and internal H_2O follows from the fact that the permeability of this cell is the same for D_2O as it is for normal water.[2] We failed to observe any deviations from normal in the cell volume within 30 sec. after immersion in D_2O. Because of the fugacity difference between heavy and light water, a normal cell immersed in D_2O would be expected to show a transient shrinkage during the period of equilibration.

The division of *Arbacia* eggs can be arrested by the immersion of the cells in D_2O at any stage of the division cycle. If the cells are placed in deuterium oxide soon after fertilization, before the appearance of the mitotic machinery, and prior to any cytologically identifiable preparations for its assembly, no such preparations take place as long as the cells remain in D_2O. Upon their return to normal sea water, an interval ordinarily elapses equal to that required for a normal cleavage, whereupon the cells divide once if the stay in D_2O has been brief or in multiples if it has been protracted. The remarkable catholicity of this antimitotic with respect to mitotic stages is made most evident, however, when it is applied after the monaster stage, that is, after the visible alterations associated with the assembly of the mitotic figure have begun. Here the cells remain arrested at exactly the stage in which they were immersed in D_2O. Furthermore, the cytological structures are stabilized so that, unlike the action of the few other postmetaphase mitotic poisons, D_2O preserves rather than destroys the achromatic figure. This seems also to be true for the cleavage furrows. Effects such as these are represented in FIGURE 1, which is a plot of

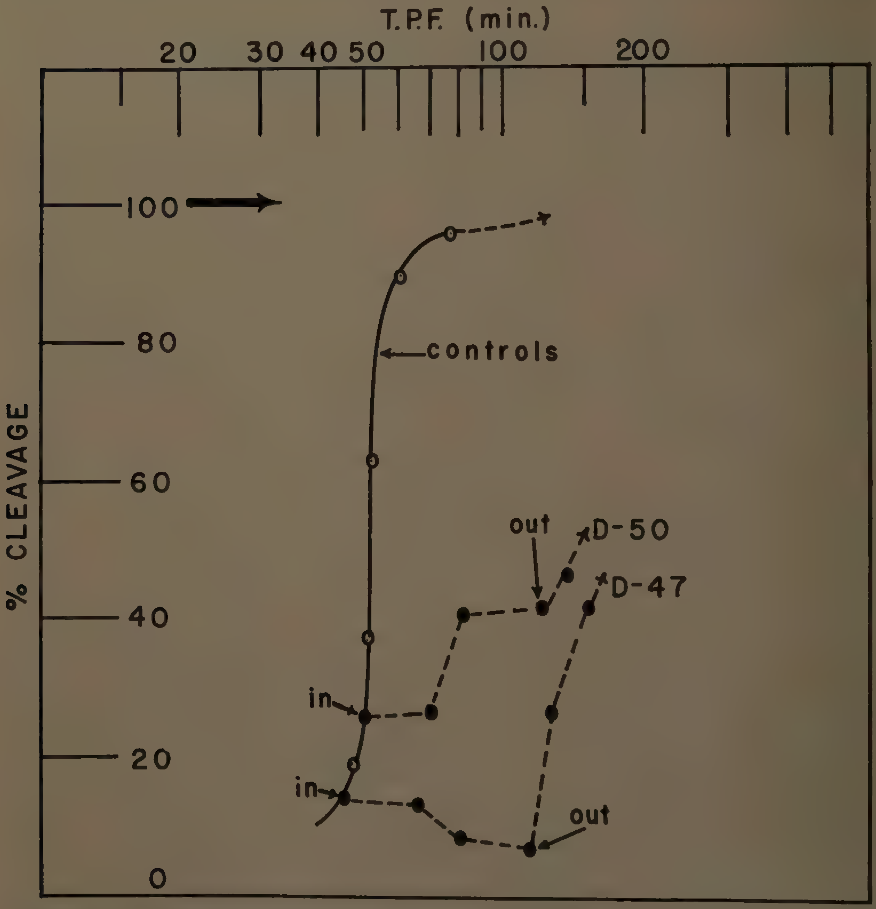

FIGURE 1. Results of an experiment in which fertilized eggs were immersed in 85 per cent D_2O-sea water 47 and 50 min. after insemination. Ordinates: per cent of population showing normal or multiple furrows. Abcissa: time postfertilization, in minutes. The abcissa is plotted logarithmically to accommodate the lengthy interval over which observations were made. Open circles: control population, cells in sea water reconstituted with normal H_2O. Filled circles: aliquots removed to sea water reconstituted to contain 85 per cent D_2O. Times of immersion and removal from D_2O are indicated as "in" and "out" on the graph. See text for fuller description.

data from an experiment in which eggs were fertilized and permitted to proceed toward normal division in ordinary reconstituted sea water. The open circles show the time course of cleavage in the control population. At 47 and 50 min. after fertilization, when approximately 15 and 30 per cent respectively of the cells *had already divided*, aliquots of the control suspension were removed and placed in 85 per cent D_2O. In the "47-minute" sample, 15 per cent of the cells had already begun to divide; because of the excellent division synchrony of the population, it is certain that most of the remaining cells were in telophase or in very late anaphase. It is most unusual for an antimitotic agent to act effectively at stages as late as these. Nevertheless, as the figure shows, no additional divisions could be counted in these eggs after their immersion in D_2O. The slight decrease in the number of countable furrows after immersion is probably not significant. For the remainder of the interval during which these cells were in heavy water, that is, 73 min., they retained the large, clear central structures identifiable as the mitotic apparatus! At 120 min. post-fertilization, the cells were washed free of D_2O, whereupon they began to cleave with a negligible delay into irregular, multicellular masses containing from three to eight "blastomeres." The activity of the cells placed in D_2O after 50 min. is the same, except that here a few cells seemed to escape the block and, at the time of removal from D_2O, there were fewer identifiable mitotic figures in the nondivided cells, suggesting that telophase nuclei had been reconstituted without cytokinesis.

The resistance of the cytoplasm to sedimentation of its denser particulates under the influence of centrifugal force is a measure of the degree of interaction among its structure-forming macromolecules and inclusions. It has been known for a long time that the "viscosity" of the cytoplasm of the sea urchin egg, measured as an inverse function of the time necessary to stratify its inclusions in a centrifugal field, is very low,[13] and that this viscosity rises by several hundred per cent prior to the appearance of the mitotic figure.[14] In order to examine the effect of D_2O on the structure-forming systems in the cytoplasm, viscosity measurements were made on *unfertilized* eggs, in which the absolute viscosity is normally no more than a few centipoise when measured at low shearing stress. Eggs were immersed in normal reconstituted sea water and in a series of media containing increasing concentrations of deuterium. The measured "viscosity" was found to rise exponentially with increasing D-enrichment so that, in 95+ per cent D_2O, the stratification time was at least twenty times longer than that required by control cells. It is to be noted that the viscosity of *pure* D_2O could not be more than 25 per cent higher than that of normal water at the temperature of the experiments.

We may now examine the five hypotheses given earlier with a view to applying them to the antimitotic effects just described.

Hypothesis No. 1 cannot be an unique explanation for the antimitotic effect of D_2O. Two features of the experimental results serve to eliminate it. First, the almost instantaneous appearance of the effect and its equally rapid reversal after replacement by normal water are inconsistent with a general depression or alteration in the pattern of metabolism. Such a mechanism would be expected to require a longer period of action before becoming manifest as a block to mitosis. Secondly, the ability of D_2O to block mitosis both before and after metaphase, and indeed at all stages of division including cytokinesis, speaks for the metabolic independence of the effect; it is quite generally assumed that whatever energy reservoirs must be filled prior to division,[8] if indeed there are any such reservoirs,[9] the postmetaphase stages of mitosis are independent of them. D_2O stops cell division at stages in which the classic inhibitors of respiratory metabolism fail.

This is not to imply that interference with metabolism is not a result of deuteration in biological systems: considerable evidence exists in the literature to support such interference. It may, furthermore, help to explain how some energy-requiring mechanical activities of cells, such as muscular contraction, are inhibited by D_2O. But metabolic depression alone cannot account for the powerful, stage-independent antimitotic action of heavy water.

Hypothesis No. 2 fails to explain the antimitotic effect for precisely the same reasons as does No. 1, which is not surprising since No. 2 is a variant of No. 1. Again, it must not be understood that its rejection with respect to antimitosis reduces the value of this mechanism as an explanation for other biological responses to deuterium. Hypothesis No. 2 may, for example, explain what appear to be altered nutritional requirements for microorganisms adapted to grow in D_2O, but even in these forms it is clear that cell division is more sensitive to deuterium than is cell growth, which leads to giant cell production in the early stages of adaptation. (The fact that some microorganisms *can* adapt to growth and division in concentrated D_2O media is another problem, the solution to which must be sought, we believe, in the differences in division mechanisms characteristic of such cells and of the cells of metazoa.)

Hypothesis No. 3 also fails because D_2O can block division after metaphase. At metaphase, all the precursors needed for the mitotic machinery and for the genetic apparatus have been recruited, the polymers assembled, and the organelles elaborated. Again it is *probable* that deuteration has some effect upon the rates of synthesis of proteins and nucleic acids, but that effect cannot operate in the blockade of mitosis after metaphase.

Hypothesis No. 4 is equivalent to the statement that D_2O is a mutagen. It should, therefore, behave as do some chemical mutagens and ionizing radiation, whose attack upon dividing cells produces mutations, chromosomal aberrations, pathological mitoses, nuclear pyknosis, and cell death. One critical test of this hypothesis is to be made in genetic experiments, where evidence of mutagenesis is easily obtained. Thus far, as it appears from the reports in this monograph, the genetic evidence is equivocal. In so far as interference with DNA replication and stability might affect chromosomal condensation and movements, this mechanism might explain mitotic inhibition as late as metaphase and somewhat beyond. However, as shown in FIGURE 1, the antimitotic effect occurs in late anaphase, telophase, and even during cytokinesis in late telophase. Since in those stages the chromosomes have long since condensed, split, separated, and perhaps vesiculated and rejoined to form the telophasic nuclei, it is necessary to conclude that the primary antimitotic effect, at least in our experiments, has occurred elsewhere.

Hypothesis No. 5 is the only one whose provisions are not directly contradicted by the data concerning mitotic poisoning in sea urchin eggs. The uniform sensitivity of all stages, the reversibility of the effect, the continuation of some replicative activity during mitotic arrest, and the remarkable stabilization of the mitotic apparatus by D_2O all support this proposal. Recast in terms of the experimental results, it would state that the deuterium effect that interferes *directly* with mitosis is a rigidification of the cytoplasm and a freezing of forming or completed organized structures, including asters, mitotic spindle, and furrows, but not including the cell centers and possibly not including chromosomal material. The exponential increase in cytoplasmic rigidity with increasing deuteration of the medium lends further support to the hypothesis.

It is interesting to consider what the molecular mechanism of such a rigidification by D_2O might be, although the expository unities forbid an extended consideration of such matters in this discussion. Suffice it to say that one possibility is that the cytoplasm as a whole, and the mitotic apparatus in particular, possess a large number of *inter*molecular H-bond cross-links. If substitution of D for H at these sites (and one is concerned with macromolecular sites, not sites on water) could result in a small increase in the strength of each cross-link, then the over-all cooperative effect could be very large indeed;[1] the "viscosity" of the system would, in fact, rise exponentially with increase in the relative concentration of D. Alternatively, the substitution of D for H in the cell water might change the hydration "spheres" surrounding macromolecules in such a direction as to permit great interaction through other types of bonds, such as salt bridges and dithio groups formed by disulfide interchange.

Recent experiments with gelatin solutions (Gross and Spindel, unpublished observations) lend credence to the H-bond cross-linking mechanism. We have found that in very dilute gelatin solutions, where the intermolecular junctions must be largely hydrogen bonds, the reduced viscosity rises much more sharply with polymer concentration in D_2O than it does in normal water. The intrinsic viscosities, however, remain approximately the same for the normal and deuterium-enriched solutions. This suggests that the molecular size and shape parameters suffer little alteration in the presence of D_2O, whereas the intermolecular attractions among the polymers in heavy water increase in strength or perhaps in number.

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Discussion

Benjamin Kaminer (*Laboratory of the Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Mass.*): On considering the mechanism of the biological effects of deuterium oxide, attention should also be given to the possibility that energy transfer or coupling mechanisms are affected by the substitution of deuterium for hydrogen. This possibility finds some support in the following findings in my own investigations on the effects of deuterium oxide on muscle.[1]

While deuterium oxide decreased markedly the force of contraction of living muscle, it did not reduce the shortening response of glycerol extracted fibers produced by ATP. Deuterium oxide retarded, however, the response of the fibers to ITP. Likewise the response to UTP was retarded but not to that of CTP. Thus deuterium oxide did not necessarily retard the final shortening process of the contractile proteins but effected rather the interaction of specific nucleotides with the actomyosin. Since the difference in molecular structure between ITP and ATP on the one hand and between UTP and CTP on the other is in the sixth position of the purine and pyrimidine rings, respectively, it would appear that reactions involving the sixth position of either ITP or UTP were retarded by deuterium.

In the case of living heart muscle, deuterium oxide again markedly reduced the force of contraction and in some instances where the contraction was not detectable, the electrical impulse continued to be propagated, that is, there was complete dissociation in the response of the electrical and mechanical events. Taken in conjunction with the observations on the glycerol extracted fibers, it seems likely that the energy transfer mechanism between the membrane and the contractile protein is retarded or blocked by the isotopic effect of deuterium.

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THE EFFECT OF DEUTERIUM OXIDE ON ASCITES TUMOR GROWTH IN MICE*

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The idea that the deuterium isotope effect may have an influence on neoplastic processes was recently revived by Katz and his associates.[1] Preliminary data were presented that encouraged the view that the presence of deuterium in substantial concentrations in a mammalian organism might differentially and adversely affect the growth of a transplanted ascites tumor in mice.[1,2] It is our purpose here to present additional data relating to these studies.

Barbour and Allen[3] in 1938 had reported that the growth of transplanted lymphosarcoma and mammary carcinoma was slower in mice drinking 40 per cent D_2O than in mice drinking ordinary water. In these experiments, the tumor-bearing deuterated mice did not live as long as their nondeuterated controls. Earlier, Fischer[4] had reported that mouse carcinoma cells in tissue culture were unable to grow when the D_2O concentration was above 50 per cent. The problem received no further attention for almost 20 years, but the recent availability of heavy water in relatively large amounts has led to a re-examination of various biological aspects of the kinetic isotope effect of deuterium.[1,5]

The studies reported here are concerned with an evaluation of the effect of deuterium oxide in body fluids on certain quantitative indices of ascites tumor growth. Measurements were also made of the serum and ascitic plasma glutamic oxalacetic transaminase (GO-T) concentrations since these and other "enzymes of injury" in the serum have been shown to be altered in experimental and clinical states in which destructive lesions of specific tissues occur.[6]

Methods

The deuterium oxide used in these experiments had been purified by triple distillation of alkaline-permanganate–treated 99.6 per cent stock material. The tritium content of the D_2O was less than 0.01 μc./gm., a concentration far below the maximum permissible level of 0.2 μc./ml.[7] Deuterium in urine was determined spectrophotometrically by analysis of the optical density in the near infrared region.[1]

Young adult female mice (CF No. 1) were deuterated by supplying them with drinking water containing deuterium oxide at various concentrations compatible with continued survival. Under such conditions, the body fluids become equilibrated with the D_2O in the drinking water fairly rapidly, and the D_2O level in plasma and urine reaches a value that is roughly 75 to 80 per cent of that in the drinking water within 7 to 10 days.[1] The concentration of deuterium in the body fluids does not reach that of the drinking water because it is continuously diluted with hydrogen derived from the food.

In the first of the two experiments reported here, two groups of mice were deuterated to 13 to 14 and to 23 to 24 atom per cent by supplying them with

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* The work described in this article was performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

drinking water of 15 and 30 atom per cent deuterium content, with a third nondeuterated group serving as a control. On the seventh day, each mouse was inoculated intraperitoneally with 7 × 10^5 Krebs-2A ascites tumor cells, and three animals of each group were sacrificed 4, 7, 9, and 14 days later. Measurements of tumor growth were made according to the quantitative methods developed by Patt *et al.*[8] A duplicate series of mice was sacrificed for the enzyme studies described below since these could not be made with the dye-stained ascitic fluid required for the tumor-growth measurements.

In the second experiment, three groups of mice were similarly deuterated to equilibrium concentrations of 13 to 14, 23 to 24, and 31 to 32 atom per cent in the body fluids by furnishing them with 15, 30, and 40 per cent D_2O, respectively, in the drinking water. A fourth group consisted of nondeuterated control mice. On the ninth day, each animal was inoculated intraperitoneally with 4.8 × 10^6 tumor cells, and mice were sacrificed 3, 5, 7, 10, and 12 days later. In this experiment, the ascitic fluid volume was determined by measuring the difference in body weight before and after the drainage and blotting up of the ascitic fluid.[9]

Glutamic oxalacetic transaminase (GO-T) concentrations in the serum and in the ascitic fluid plasma were determined spectrophotometrically according to the method of Steinberg *et al.*[10] Samples of these fluids were preserved at −10° C. and were analyzed within 3 weeks for transaminase levels.

Mitotic indices were determined in the second experiment by examining at least 1000 tumor cells in the ascitic fluid smear of each animal when it was sacrificed. These smears were immersed in ether alcohol for 30 min., stored in 70 per cent alcohol, and stained with hematoxylin and eosin.

Results and Discussion

Growth of ascites tumors. The results of the first experiment are shown in TABLE 1, which gives the mean values for various quantitative indices of tumor growth. Mean total ascitic fluid volume, mean total tumor-cell population, and mean total tumor-cell volume increased steadily among the control animals until after the ninth day. The decline observed on the 14th day reflects the illness and the moribund state of the mice. By the seventh day, there was an inverse relationship between the degree of deuteration and the extent of tumor growth. If the total tumor-cell population be taken as the key index to tumor growth, its value on the seventh day in animals drinking 30 per cent D_2O is 56.9 per cent of that of the control group, while in the mice drinking 15 per cent D_2O, the comparable value is 73.8 per cent. By the ninth day, these proportions reach 27.5 and 58.5 per cent, respectively. The other indices show similar and consistent relationships.

The tumor-growth data from the second, and larger, experiment are given in TABLE 2. Here the inoculum was 6.8 times larger than in the earlier experiment, and the time scale of tumor growth was accordingly altered. Again, the means of the total ascitic fluid volume, the total tumor-cell population, and the total tumor-cell volume showed changes consistent with an adverse effect of deuteration on tumor growth. Thus, by the fifth day, the mean total tumor-cell populations in mice deuterated to 13 to 14, 23 to 24, and 31 to 32 atom per cent were 83.2, 50.5, and 33.2 per cent, respectively, of the tumor

TABLE 1

GROWTH CHARACTERISTICS OF KREBS-2A ASCITES TUMOR IN DEUTERATED AND NONDEUTERATED MICE[*]

Day	Deuterium in body fluids (atom %)	Number of mice	Mean total ascitic fluid volume (ml.)	Mean total tumor-cell volume[†] (ml.)	Mean total tumor-cell population (10^6 cells)
4	23–24	3	3.10	0.72	183.8
	13–14	3	2.86	0.68	202.9
	0	1	2.17	0.51	149.4
7	23–24	2	3.85	1.55	357.9
	13–14	3	5.41	1.97	464.7
	0	3	6.86	2.07	629.8
9	23–24	3	5.14	2.81	340.1
	13–14	2	9.44	4.38	723.6
	0	3	14.33	7.75	1238.6
14	23–24	3	6.50	7.74	422.6
	13–14	1	12.00	4.88	884.4
	0	2	11.50	2.59	791.7

[*] Inoculated intraperitoneally with 7×10^5 tumor cells on the seventh day of deuteration.

[†] Mean tumor-cell volume is derived by multiplying the total ascitic fluid volume by the per cent packed-cell volume (with a correction for the dilution by the dye used in the procedure for volume determination).

TABLE 2

GROWTH CHARACTERISTICS OF KREBS-2A ASCITES TUMOR IN DEUTERATED AND NONDEUTERATED MICE[*]

Day	Deuterium in body fluids (atom %)	Number of mice	Mean total ascitic fluid volume (ml.)	Mean total tumor-cell volume (ml.)	Mean total tumor-cell population[†] (10^6 cells)
3	31–32	5	1.09	0.22	60.15 ± 8.46
	23–24	10	1.19	0.21	63.61 ± 7.28
	13–14	10	1.05	0.20	67.21 ± 7.17
	0	10	0.98	0.19	64.40 ± 7.28
5	31–32	5	1.79	0.39	98.00 ± 13.12
	23–24	8	2.45	0.39	148.94 ± 12.42
	13–14	10	4.02	0.80	245.28 ± 15.43
	0	9	3.95	0.73	294.83 ± 14.49
7	31–32	5	2.73	0.69	171.55 ± 16.67
	23–24	5	2.49	0.73	182.66 ± 20.07
	13–14	5	2.83	1.17	197.10 ± 32.05
	0	5	5.08	1.78	505.22 ± 81.78
10	23–24	4	2.40	0.61	152.80 ± 21.54
	13–14	4	2.48	1.05	286.36 ± 85.13
	0	5	7.89	2.29	724.86 ± 100.05
12	13–14	1	1.96	0.82	264.99
	0	4	12.33	3.38	955.01 ± 173.13

[*] Inoculated intraperitoneally with 4.8×10^6 tumor cells on the ninth day of deuteration.

[†] Mean total tumor-cell population ± standard error of the mean.

populations in nondeuterated control mice. Comparable percentage values for the seventh day were 39.0, 36.2, and 34.0.

These results rather clearly demonstrate an effect on ascites tumor-cell growth associated with the presence of deuterium in the body fluids. Although the tumor is not destroyed, its growth rate is substantially reduced, and the extent of this effect roughly corresponds to the degree of substitution of deuterium for hydrogen in the organism.

Glutamic oxalacetic transaminase studies. The effect of deuterium on ascites-tumor growth can be postulated to be a result of an inhibition of cell division, or of a destruction of individual tumor cells, or of a combination of both processes. It was thought that some insight into this matter might be afforded by an examination of the glutamic oxalacetic transaminase (GO-T) levels in the ascitic fluid plasma and in the blood serum of the tumor-bearing mice. In an earlier paper[1] we noted that the serum GO-T values were normal in mice in which the ascites tumor grew subcutaneously in a solid phase and that the GO-T values for normal mouse sera ranged from 20 to 120 arbitrary units.

TABLE 3

GLUTAMIC OXALACETIC TRANSAMINASE (GO-T)* VALUES IN BLOOD SERUM AND ASCITIC FLUID IN DEUTERATED AND NONDEUTERATED MICE INOCULATED WITH KREBS-2A ASCITES TUMOR†

Day after inoculation	Deuterium in body fluids (atom%)	No. of mice	Mean serum GO-T	No. of mice	Mean ascitic fluid GO-T	Ratio: (SGO-T/AGO-T)
4	23–24	3	145	2	173	0.84
	13–14	3	79	3	169	0.47
	0	3	197	3	112	1.76
7	23–24	3	405	3	350	1.16
	13–14	3	193	2	333	0.58
	0	3	289	2	418	0.69
9	23–24	3	333	2	681	0.49
	13–14	3	377	1	688	0.54
	0	3	298	3	326	0.91
14	23–24	3	713	3	1311	0.54
	13–14	3	479	2	301	1.59
	0	3	534	3	263	2.03

* GO-T concentrations are given in arbitrary units defined by Steinberg *et al.*[10]

† Inoculated intraperitoneally with 7×10^6 tumor cells on the seventh day of deuteration.

TABLE 3 presents the GO-T values in blood serum (SGO-T) and in ascitic fluid plasma (AGO-T) in mice from the first experiment. These transaminase levels were found in mice that were sacrificed at the same time as those used for the determination of the tumor-growth characteristics. Mice that were deuterated but not inoculated with the ascites tumor had serum GO-T values that ranged from 30 to 91 arbitrary units, a span that is well within the normal limits. The tumor-bearing mice showed elevated serum GO-T values and even more elevated ascitic plasma GO-T levels. With one exception (the 23–24 per cent deuterium group on the seventh day), the SGO-T/AGO-T ratio on any day was greatest in the nondeuterated animals and was least in the most highly deuterated mice. Within each treatment group, the mean SGO-T level gradually increased with time, and the AGO-T levels increased even more markedly.

Similar results were obtained with mice in the second experiment and are summarized in TABLE 4. Again, the relationships are similar except for an unaccountable reversal on the seventh day. If GO-T values can be used as a measure of cell destruction, these data suggest that deuterium at appropriate levels in the body fluids, including the ascitic plasma, leads to tumor-cell destruction.

TABLE 4

GLUTAMIC OXALACETIC TRANSAMINASE VALUES (GO-T)* IN BLOOD SERUM AND ASCITIC FLUID IN DEUTERATED AND NONDEUTERATED MICE INOCULATED WITH KREBS-2A ASCITES TUMOR†

Day after inoculation	Deuterium in body fluids (atom %)	No. of mice	Mean serum GO-T	No. of mice	Mean ascitic fluid GO-T	Ratio:	
						SGO-T/AGO-T	AGO-T/ 10^6 tumor cells
3	31–32	5	117	5	294	0.40	5.32
	23–24	10	100	10	235	0.42	4.43
	13–14	10	118	10	240	0.49	3.75
	0	10	132	10	206	0.64	3.13
5	31–32	6	253	6	400	0.63	7.30
	23–24	10	155	10	222	0.70	3.65
	13–14	10	144	10	192	0.75	3.15
	0	10	143	9	204	0.70	2.73
7	31–32	5	1236	5	713	1.73	11.43
	23–24	5	838	4	688	1.22	9.40
	13–14	5	480	5	518	0.93	7.44
	0	5	246	5	254	0.97	2.56
10	23–24	5	850	4	1031	0.82	16.42
	13–14	5	492	3	564	0.87	4.90
	0	5	220	5	175	1.26	1.91
12	13–14	2	406	1	924	0.44	6.83
	0	5	208	5	409	0.51	5.28

* GO-T concentrations are given in arbitrary units defined by Steinberg *et al.*[10]

† Inoculated with 4.8 × 10^6 tumor cells on the ninth day of deuteration.

This conclusion is reinforced by a consideration of the amount of AGO-T per tumor cell. This ratio can be calculated by dividing the mean total amount of AGO-T (arrived at by multiplying the mean AGO-T concentration by the mean total ascitic fluid volume) by the mean total tumor-cell population. Such data for the second experiment, derived from TABLES 2 and 4, are given in TABLE 4. These indices declined slowly with tumor growth in the nondeuterated control mice while they increased strikingly in the two highly deuterated groups. This calculation may be criticized because, if AGO-T is released into ascitic plasma by cells that are injured or destroyed, it is not reasonable to relate the AGO-T level to the number of cells that remain. On the other hand, if no cell injury were involved, the calculated ratios should be approximately constant, or they should decline slowly, as do those of the control mice.

Nonviable cells. Examination of eosin-treated ascitic fluid for stained tumor cells offers another method for ascertaining cell death since resistance to eosin staining has been used as an indication of viability.[8,12] TABLE 5 gives the percentage of stained tumor cells in the second experiment, and it can be seen that their incidence rose fairly steadily as the tumor grew in deuterated mice. The control mice showed no such trend, and the incidence of stained cells was within normal limits except on the seventh day. This exception may have resulted from a technical error, and it may be related to the peculiar reversal of SGO-T/AGO-T ratios in these animals on the same day.

TABLE 5

INCIDENCE OF EOSIN-STAINED (NONVIABLE) TUMOR CELLS AND OF MITOTIC CELLS IN ASCITIC FLUID OF MICE IN TABLE 2

Deuterium in body fluids (atom %)	Day	No. of mice	Mean per cent eosin-stained cells	Mean per cent mitotic cells
31–32	3	5	1.93	2.64
	5	6	0.98	2.90
	7	5	6.92	0.98
23–24	3	10	3.08	3.06
	5	10	1.36	2.46
	7	5	5.07	1.26
	10	4	11.72	2.00
13–14	3	10	2.71	2.44
	5	10	2.77	1.72
	7	5	6.34	2.20
	10	5	8.56	2.40
	12	2	7.17	—
0	3	10	2.02	3.10
	5	9	1.84	2.50
	7	5	5.80	3.50
	10	5	2.86	2.96
	12	5	2.81	2.10

Although there was a late increase in nonviable cells associated with deuteration, one might have anticipated even larger percentages in the highly deuterated mice where tumor growth was inhibited fairly early. In other words, the detectable presence of nonviable cells did not keep pace with the inhibition of tumor-cell growth nor with the enzyme evidence of injury to the tumor cells.

Mitotic index. It is quite possible that the depressing effect of deuterium on ascites tumor growth may be the result of interference with some phase of cell division. Interference with mitosis has been invoked as an explanation of increased survival time of deuterated mice inoculated with Ehrlich mouse ascites tumor,[13] and it has been described in the early development of deuterated sea urchin eggs (P. R. Gross and W. Spindel, elsewhere in this monograph). The mitotic indices in the stained smears of ascitic fluid made in our second experiment are given in TABLE 5. There were no significant differences in these values among the groups, nor was any secular trend discernible. It would appear that, at least in the case of the situation discussed in this paper, the deuterium effect is accomplished mainly by some injury or destruction of the ascites tumor cell rather than by suppression of cell division, although both mechanisms may be operating at the same time.

Summary and Conclusions

The growth of Krebs-2A ascites tumors in mice was appreciably depressed by the presence of deuterium in the body fluids in concentrations ranging from 13 to 32 per cent. Measurement of the glutamic oxalacetic transaminase (GO-T) levels revealed that with increasing deuteration there was a progressive elevation of serum GO-T in tumor-bearing mice and an even more rapid rise of ascitic plasma GO-T. These data suggest that deuteration of the organism leads to tumor-cell injury with resulting release of GO-T into the ascitic fluid, whence it is carried into the general circulation. The notion that deuterium injures and possibly destroys mouse ascites tumor cells is supported, in part, by the terminal increase in the incidence of eosin-stainable tumor cells, and, in part, by the absence of any striking difference in mitotic index of deuterated and nondeuterated tumor cells. These conclusions properly apply to transplantable mouse ascites tumors, and they may or may not be applicable to other biological material.

Acknowledgments

We express our thanks to J. J. Katz for the supply of deuterium oxide and for his stimulating interest in this problem; to Robert L. Straube for his generous aid in supplying strains of the ascites tumors that were used; and to Betty Van Dolah for her technical assistance in portions of this study.

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FURTHER STUDIES ON STERILITY PRODUCED IN MALE MICE BY DEUTERIUM OXIDE

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Introduction

We have previously reported that deuterium oxide in the drinking water of either male or female mice produces sterility.[1,2,†] An investigation of some of the conditions—with particular reference to time—of deuterium oxide treatment to produce sterile C_{57} male mice indicated that the sensitive phase of sperm production centered around the late prophase of meiosis. In some experiments, although D_2O was almost completely absent during maturation of the sperm, and when the mice mated, these sperm exhibited the effects of their much earlier contact with D_2O. No viable offspring were obtained from these matings. We concluded that the presence of D_2O during the late prophase and meiotic divisions interfered with the normal construction or division (or both) of genetic material. It was suggested that changes in the forces, principally hydrogen bonds, in macromolecules affected their structural characteristics and resulted in abnormal division.

The objective of the experiments reported here was to determine the phases of embryonic development of the mouse at which the lethal action of deuterium oxide on sperm is manifested. These investigations on embryonic growth initiated by sperm developed in D_2O have yielded additional evidence that D_2O severely damages the genetic material of developing sperm, with resulting sterility of the male mouse.

Methods and Results

"Dominant lethal" experiments. Male and female Swiss mice were used.‡ The fertility of the experimental and control male mice was determined by mating each with 2 or 3 female mice prior to the administration of D_2O to the experimental male mice. Only initially fertile mice were used in subsequent experiments. The 15 experimental male mice were then supplied with drinking water containing 30 per cent D_2O in lieu of tap water for the duration of the experiment. The males were 2½ to 3 months old at this time. At weekly intervals, beginning 2 weeks after the first administration of D_2O, 3 (later 4) normal virgin female mice 3 months old were placed with each male. Ten control males supplied with tap water instead of deuterated water, but otherwise maintained under identical conditions, were mated at the same time. Each morning any female that had copulated (as indicated by the presence of a

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† It is shown in this paper that the term sterility accurately connotes the effect in the male mouse. Czajka and Finkel (elsewhere in this monograph) have suggested "loss of reproductive potential" to describe the effect of D_2O upon the fertility of mice. This phrase is more descriptive of the effect of D_2O in the female mouse.

‡ These mice were obtained from Simonsen Laboratories, Gilroy, Calif.

vaginal plug) was removed and housed in a separate cage. After 1 week, all females were removed and replaced. By this procedure the female mice received a minimal exposure to deuterated water, never longer than 1 week, and in many instances only 1 or 2 days.

Sixteen or 17 days after the vaginal plug was observed, or—if no vaginal plug was observed—16 days after removal from the male mouse cage, the female mice were sacrificed. They were examined for viable embryos and for dead embryos or resorption sites (regressions).[3]

TABLE 1

EFFECT OF 30 PER CENT D_2O IN DRINKING WATER ON FERTILITY OF MALE MICE

	Time of treatment prior to mating (weeks)								
	D_2O-treated males (15)				Control males (10)				
	2	3	4	6*	2	3	4	6*	Totals 2 to 6
Mice with vaginal plugs									
No. of females	24	23	23	17	16	11	11	8	46
No. pregnant	23	16	11	4	15	9	11	8	43
No. embryos	246	107	66	19	148	75	132	87	442
No. regressions	15	12	6	0	15	16	9	10	50
Regressions (%)	5.7	10.1	8.3	0	9.2	17.6	6.4	10.3	10.2
Embryos/pregnancy	10.7	6.7	6.0	4.8	9.9	8.3	12.0	10.9	10.3
Mice without vaginal plugs									
No. of females	21	22	21	43	14	19	19	30	82
No. pregnant	11	12	6	3	10	15	12	23	60
No. embryos	84	76	42	5	77	138	119	197	531
No. regressions	11	12	7	8	12	15	6	19	52
Regressions (%)	11.6	13.6	14.3	75	13.5	9.8	4.8	8.8	8.9
Embryos/pregnancy	7.6	6.3	7.0	1.7	7.7	9.2	9.9	8.6	8.9
Combined									
No. of females	45	45	44	60	30	30	30	38	128
Total No. pregnant	34	28	17	7	25	24	23	31	103
Pregnant (%)	76	62	39	12	83	80	76	82	80
Total No. embryos	330	183	108	24	225	213	251	284	973
Total No. regressions	26	24	13	8	27	31	15	29	102
Regressions (%)	7.3	11.6	10.7	25	10.7	12.7	5.6	9.3	9.5
Embryos/pregnancy	9.7	6.5	6.4	3.4	9.0	8.9	10.9	9.2	9.4
Embryos/mating	7.3	4.1	2.5	0.4	7.5	7.1	8.4	7.5	7.6

* Four females/male.

Results of "dominant lethal" experiments. The results are summarized in TABLE 1. The data on the experimental and control mice in each group have been subdivided according to whether or not a vaginal plug was observed.

No increase in the number of regressions was observed (the high percentage of regressions obtained after 6 weeks of D_2O treatment is due to 1 female mouse, which had 7 regressions). After 2 weeks of treatment with D_2O, no significant decrease in the percentage of pregnant females, or the litter size, was noted. However, beginning 3 weeks after administration of D_2O a progressive decrease in both the percentage of pregnancies and the embryos per pregnancy was observed, until after 6 weeks of treatment with D_2O, only 12 per cent of the females became pregnant, and the average number of embryos per pregnancy was 3.4. This compares with the over-all pregnancy ratio of 80 per cent and 9.4 embryos per pregnancy observed in the control mice. Embryos per mating with the D_2O-treated mice had dropped to 5 per cent of that of the controls after 6 weeks (0.4 embryo per mating compared to 7.6 embryos per mating for the control).

Since each male mouse was mated with 3 (or 4) female mice, the number of pregnant females obtained each week from each individual male can also be determined. This is shown in TABLE 2. After 2 weeks of administration of D_2O, 13 of the 15 experimental mice had either 2 or 3 fertile matings (out of 3 possible), and only 1 male mouse had no fertile matings (the following week he was fertile). This may be compared with the observations made at 6 weeks, when only 2 of the 15 males had 2 fertile matings (out of 4 possible) and 10 of the males did not have a fertile mating.

TABLE 2
FREQUENCY OF FERTILE MATINGS OF D_2O-TREATED MALE AND NORMAL FEMALE MICE*

	Duration of treatment (weeks)	No. pregnant females/male (N)				
		4	3	2	1	0
		No. of males associated with N pregnant females				
		Control male mice (10 total)				
H_2O	2	—	5	5	0	0
	3	—	4	6	0	0
	4	—	4	5	1	0
	6	4	4	0	1	0
		D_2O male mice (15 total)				
D_2O	2	—	7	6	1	1
	3	—	4	6	3	2
	4	—	2	5	2	6
	6	0	0	2	3	10

* Three female mice were mated with each male except at 6 weeks, when 4 female mice were mated with each male.

The results of this experiment strongly suggest that sperm produced in the presence of D_2O are either so defective that they are unable to penetrate the egg* or that, upon entering the egg, fertilization occurs, but damaged genetic material leads to early death of the fertilized mouse egg.

Observations of Development of Mouse Eggs Fertilized by D_2O-treated Males

Eight male Swiss mice 3 months of age from the Lawrence Radiation Laboratory colony were provided with drinking water containing 30 per cent D_2O in

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* The presence of vaginal plugs indicates that copulation occurred by D_2O-treated animals. Sperm motility is presumably not an important factor in the transport of sperm through the uterus and ovarian tubes.[4]

TABLE 3

STAGE AND CONDITION OF EGGS FROM MICE FERTILIZED BY NORMAL OR D_2O-TREATED MALES

	No. females	Time after copulation (days)	Females without eggs	Condition of eggs	Stage of eggs (No. of cells)					Total eggs
					1	2	3	4	8 or more	
Control males	5	1½ to 2	1	N*	3	4	3	2	25	37
				D*	0	0	0	0	0	0
D_2O-treated males	3	1½ to 2	0	N	23	4	1	0	0	28
				D	1	1	0	0	0	2
Control males	5	2½ to 3	1	N	0	0	0	0	28	28
				D	1	1	0	0	0	2
D_2O-treated males	9	2½ to 3	2	N	9	4	0	0	4	17
				D	1	6	2	1	33	43

* N = normal, D = degenerating.

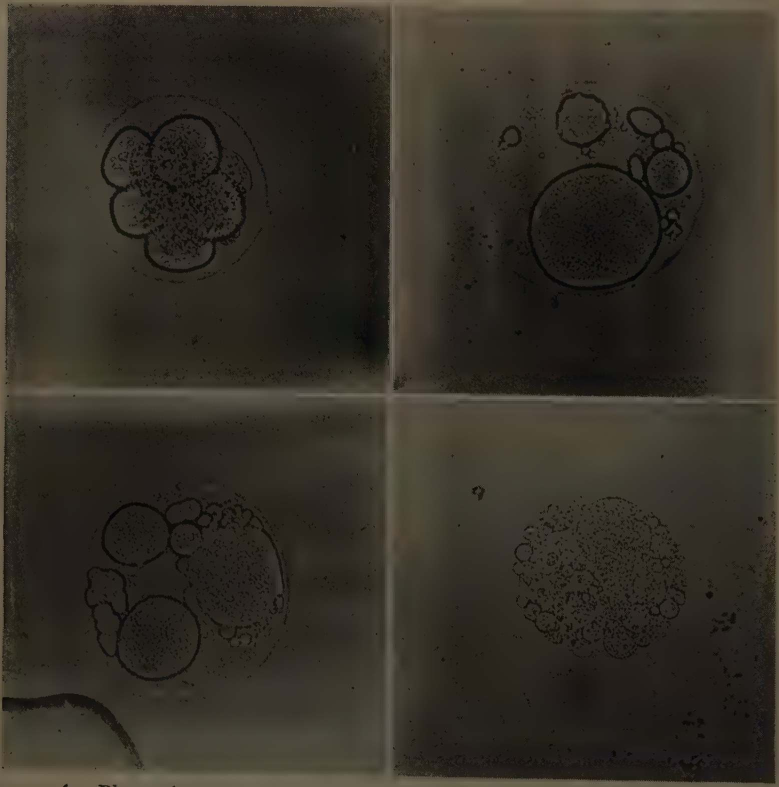

FIGURE 1. Photomicrographs of eggs obtained from 2 to 2½ days after copulation. Upper left is an example of a normal 8-cell egg. Upper right and both lower pictures are examples of abnormal cell types resulting from mating of D_2O-treated males with normal female mice. ×250.

lieu of tap water for 5½ to 6 weeks. The male mice were placed at midnight in cages with virgin female mice in estrus. At 10:00 A.M. the following day the females were removed and the presence of vaginal plugs determined. Thus the time of fertilization was known within 10 hours. Normal control mice were mated in the same fashion. At the desired time after copulation, the female mice were sacrificed, and the eggs flushed from each oviduct with 0.9 per cent NaCl and observed under magnification up to 250× after staining with acridine orange (1 to 40,000 in 0.9 per cent NaCl and 0.01 *M* phosphate buffer, *p*H 7.4). The results are summarized in TABLE 3.

In general, 55 to 65 hours after fertilization, most control mice had numerous eggs in the multicell stage (8 to 16 cells). Relatively few 2- or 4-cell eggs, or disintegrating eggs, were observed. However, a high frequency of abnormal eggs was obtained from females mated with D_2O-treated mice; many were 1, 2, or 4 cells. The cells were often not of uniform size, and in many instances they were not congruent. A few of the aberrant cell types are shown in FIGURE 1. These aberrant cells can be compared with examples of degenerating cells found in a study by Lewis and Wright.[5]

After the experimental males had been provided with 30 per cent D_2O for 7 weeks, they were returned to ordinary water. Two weeks later these mice were again mated with normal females, and the eggs were examined approximately 60 hours after copulation. Again, a high frequency of degenerating eggs was observed.

Discussion

A reduced number of litters and fewer mice per litter are obtained when male Swiss mice that have been maintained on 30 per cent D_2O in lieu of tap water are mated with normal female Swiss mice. Nearly complete sterility is obtained when Swiss male mice are maintained on 30 per cent D_2O for 6 weeks. At this time, although many broken and nonmotile sperm are present in the vas deferens, 20,000 to 30,000 motile sperm were observed in each vas deferens of 2 sterile male mice that were examined, confirming a similar observation on C_{57} mice.[2] It is extremely unlikely that the degeneration of the eggs could have resulted from the mere presence of 20 to 30 per cent D_2O in the sperm, since a sperm constitutes an extremely small fraction (much less than 0.1 per cent) of the total mass of a fertilized egg. In addition, the gross sterility observations have shown that sterile matings result from male mice that have been returned to normal water for at least 2 weeks after a 2-week treatment with 30 per cent D_2O.[2] In the present study we found degenerating eggs when male mice that had been on D_2O for 7 weeks and then had been returned to normal water for 2 weeks were mated with normal females. The sperm of mice returned to normal water for 2 weeks certainly have very little deuterium in them.

Our experiments have not shown that the sperm have entered the eggs. The observation of motile sperm would suggest that the sperm would be capable of entering the eggs. Such sperm, if abnormal in genetic complement, could lead to subsequent divisions that would be abnormal and that in many instances would not proceed past the one- to four-cell stage. Experiments to determine whether the sperm do enter the egg and whether fusion of the pronuclei does occur are in progress.

Our experiments indicate no increase in embryonic deaths after implantation. This is in marked contrast to the effects of radiation in which a large increase in such deaths, termed "dominant lethal mutation," occurs.[3] This suggests that the damage to the genetic material caused by D_2O differs from damage caused by radiation.

Hermans *et al.*[6,7] and Scheraga (elsewhere in this monograph) have shown that the substitution of D for H in macromolecules affects the thermodynamics of the helix-coil transformation. With ribonuclease the temperature required for the helix-coil transition is raised by deuteration. Crespi *et al.* (elsewhere in this monograph) have reported that elevated temperatures are necessary for growth of certain microorganisms in D_2O, which is consistent with the hypothesis that the presence of D_2O leads to an increased stability of the helix. Gross and Spindel[8] (elsewhere in these pages) have shown that D_2O causes a reversible inhibition of mitosis of *Arbacia punctulata*. In addition, they have demonstrated an increased viscosity of gelatin solutions as well as a higher liquefaction temperature in the presence of D_2O (elsewhere in this monograph). Previous experiments indicated that the phase of sperm production most sensitive to D_2O is the meiotic phase. Sperm that have undergone meiotic division in the presence of D_2O during their genesis are not capable of producing offspring even though the animal is not subsequently supplied with D_2O.

At this stage of sperm production, chromosomal reorganization occurs. It is suggested that the presence of deuterium markedly affects this process, principally by changes in the forces of association of macromolecules. Motile sperm are obtained, but the genetic material is so abnormal that although fertilization of the egg may occur, its death soon follows. Experiments are in progress to further characterize changes in the genetic material of sperm resulting from the presence of D_2O.

Summary

Thirty per cent D_2O in the drinking water of male Swiss mice produces sterility. Both a decrease in litter size and a decreased percentage of fertile matings were observed when treated males were mated with normal females. No increase in regressions was obtained. The development of mouse eggs in a female mated with a D_2O-treated male is generally abnormal; few multicelled eggs are obtained 2 to 2½ days after copulation. It is suggested that changes in physical forces that bind macromolecules are produced by the substitution of deuterium for hydrogen. These changes may result in abnormal genetic material in developing sperm.

Acknowledgments

We are grateful to T. P. Lin, Department of Anatomy, University of California Medical School, San Francisco, Calif., for demonstrating the technique for flushing the mouse eggs from the oviducts. T. P. Lin and Laurel Glass (of the same department) also assisted us in interpreting the photomicrographs of the eggs.

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EFFECT OF DEUTERIUM OXIDE ON THE REPRODUCTIVE POTENTIAL OF MICE*

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That deuterium oxide (heavy water) has many biological effects that are strikingly different from those of ordinary water has been appreciated for almost 25 years.[1,2] The recent availability of D_2O has led to a renewal of interest in a number of properties, among which is its depressant action on many living systems.[3] Because deuterium is effective in retarding the growth of a rapidly growing, transplantable ascites tumor in mice,[4] we examined some of the effects of D_2O on another rapidly growing system, the mouse embryo.

In a pilot study a litter of 2 male and 5 female mice, caged together, had been started on 25 atom per cent deuterium in the drinking water at 7 weeks of age. Within 3 months each female had been pregnant twice, but no live offspring resulted. It was not known whether the young had been viable at birth and destroyed later, or whether they had been born dead. These observations led to some systematic experiments in which deuteration of female mice was instituted at various times before and after mating in order to study the effects upon pregnancy and upon the viability of the newborn.

Methods

The deuterium oxide used in this study was triple distilled from 99.6 per cent stock, and its tritium content was less than 0.01 μc./gm.[3] The deuterium was administered to the mice in the drinking water at a concentration of 25 atom per cent in the first two experiments and at 10, 20, and 30 atom per cent in the third. Curves showing the course of deuteration of mice drinking heavy water of various concentrations have been given in previous papers.[3,5]

In the first of the three sets of experiments, the length of time on deuterium was varied in relation to the mating period (FIGURE 1). Six groups of CF No. 1 female mice were placed on the following schedule: (A) control group, no deuterium; (B) deuterium oxide begun 3 weeks after a male mouse had been introduced into the cage; (C) D_2O started 2 weeks after a male was introduced; (D) 1 week after a male was introduced; (E) the day a male was introduced; and (F) 7 days prior to the introduction of a male that had also been on deuterium for 1 week. The mice were 3 months old at the beginning of the experiment, and the females were housed 10 to a cage. Each male remained with a group of females for 7 days and pregnant females were isolated 2½ weeks after mating was started. The nonpregnant females were rebred to a fresh male 3 weeks after the beginning of the first mating period and were transferred to the appropriate deuteration group. Nonpregnant females from Group B, for example, were started on deuterium on the first day of the second mating period and were henceforth properly considered to be in Group E. In this way the results were combined with those of the earlier mating groups of equiv-

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* The work described in this article was performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

alent duration of deuteration. The general scheme is given in the left side of FIGURE 1.

In order to appraise the reversibility of the findings with return of the mice to ordinary water intake, the administration of deuterium was terminated in one half of the animals in Groups B, C, D, and E. This change to ordinary water took place in Group E on the first day of the new mating period; it occurred in Group D 7 days later, in Group C 14 days later, and in Group B 21 days later. These procedures are indicated graphically in the right-hand portion of FIGURE 1.

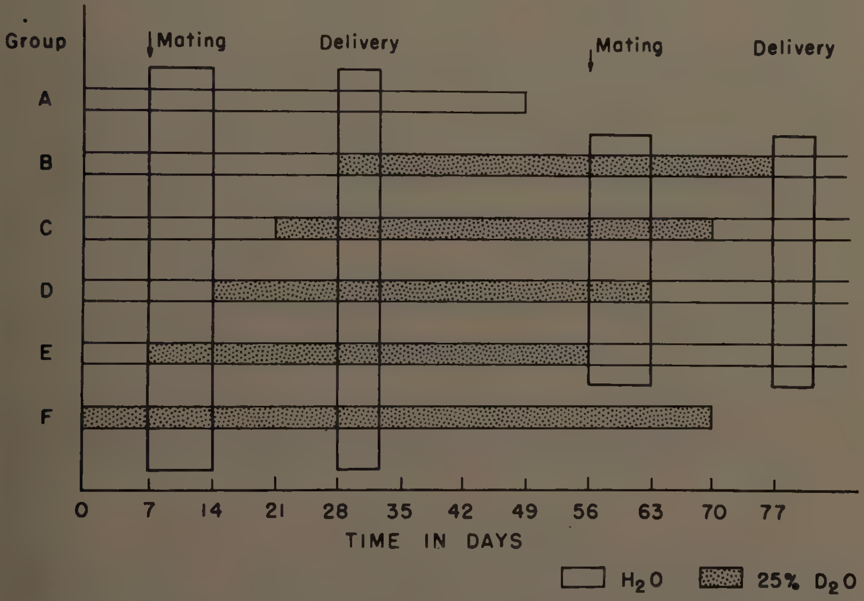

FIGURE 1. Diagram of Experiment 1. Shaded bars indicate period of deuteration of female mice with 25 per cent D_2O.

Pregnancies were detected by palpation and rapid weight gain during the week prior to expected delivery. In the absence of newly born mice, delivery was assumed to have occurred if there was a sudden weight loss of at least 6 gm. after a previous rapid weight gain.

A second set of experiments with 5-month-old CF No. 1 mice was designed to check on the status of the fetuses *in utero*. Groups of females were subjected to a staggered arrangement of deuteration similar to that employed in the first series: Group A, 3 cages of 10 mice each, no deuterium; Groups B through F, 10 mice each started on 25 atom per cent deuterium 4, 11, 18, 25, and 32 days before they were sacrificed (FIGURE 2). During the mating period, the males were replaced daily in each cage for 5 successive days. This procedure was designed to minimize deuterium uptake by the males since the body fluids of mice become deuterated only to a level of 8 to 9 per cent after the first 24 hours on 25 per cent D_2O in the drinking water.[5] The female mice were all sacrificed

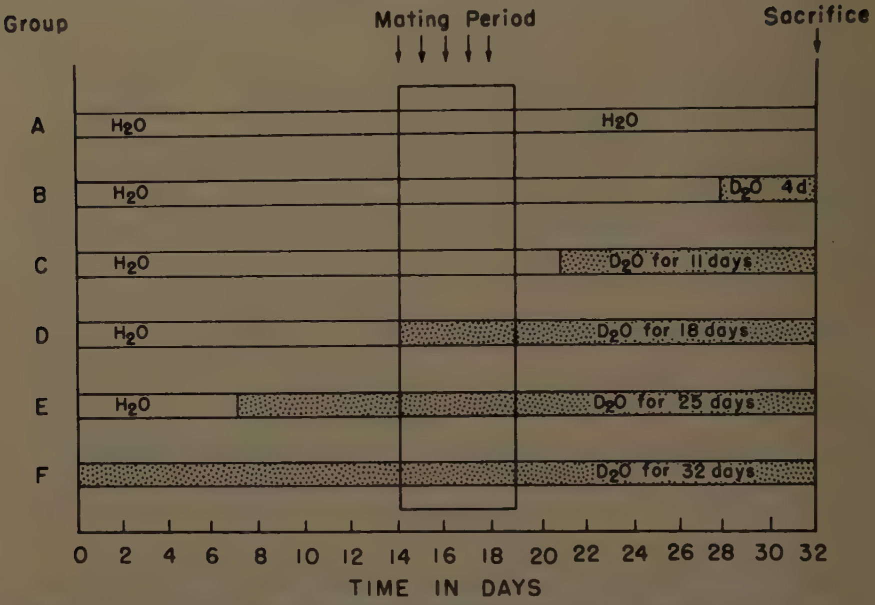

FIGURE 2. Diagram of Experiment 2. Shaded bars indicate period of deuteration of female mice with 25 per cent D_2O. Arrows indicate days when fresh male was introduced into breeding cage.

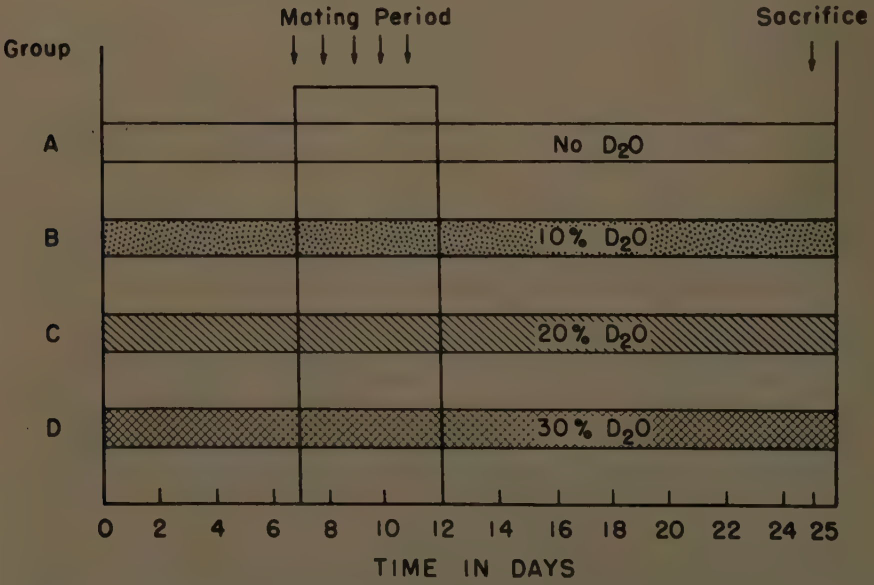

FIGURE 3. Diagram of Experiment 3. Marked bars indicate period of administration of D_2O of indicated concentrations in drinking water. Arrows indicate days when fresh male was introduced into breeding cage.

on the 18th day after the first male had been introduced into the cage, that is, on the 13th to 18th day of pregnancy if successful mating took place. The uterus of each sacrificed mouse was examined for the presence of live and dead fetuses.

The third set of experiments involved a series of mice placed on drinking water of varying D_2O content 1 week before mating (FIGURE 3). Except for the differences in D_2O level, the mice were comparable to Group E in the second experiment (FIGURE 2). Males were replaced daily during the 5-day mating period, and again the female mice were sacrificed on the 18th day for examination of the uterine contents.

TABLE 1

EFFECTS OF DEUTERIUM OXIDE ON PREGNANCY IN MICE*

Group	No. days on D_2O before birth	No. mice	No. breeding trials	No. pregnancies	Pregnant per breeding trial (%)	Pregnant mice (%)	Number of litters: No newborn found	Number of litters: All newborn dead	Number of litters: At least one newborn alive	Nonviable litters (%)	Total No. observed newborn	Observed mean litter size	No. alive at 30 days: ♂	No. alive at 30 days: ♀
A	—	20	23	15	65.2	75	0	1	14	6.7	111	7.4	44	34
B	0–3	20	32	13	40.6	65	1	2	10	23.1	72	6.0	11	15
C	7–10	20	19	11	57.9	55	9	0	2	81.8	19	9.5	0	0
D	14–17	20	18	8	44.4	40	8	0	0	100.0	0	0	—	—
E*	21–24	30	29	10	34.4	33.3	7	2	1	90.0	6	2.0	0	0
F*	28–31	26	24	4	16.7	15.4	3	1	0	100.0	0	0	—	—

* Males also deuterated.

Results

When the total pregnancy experience during and after deuteration was tabulated for each mouse included in the first series of experiments, it was found that the number of animals in each group that had a history of at least one pregnancy was as follows: Group A, 14 (70 per cent); Group B, 16 (80 per cent); Group C, 13 (65 per cent); Group D, 13 (65 per cent); and Group E, 12 (60 per cent). These data indicate that the groups of mice were nearly uniformly fertile, and that the potentiality for becoming pregnant was fairly evenly distributed among the several groups of mice.

TABLE 1 gives the results of deuteration on pregnancy in the first part of the first set of experiments. Only positively diagnosed near-term pregnancies have been included. Since all the members of one litter of Group E were alive on the day of delivery, it is probable that a few of the animals in Group D (in which deuteration was started 1 week later) were viable at birth. It appears from TABLE 1 that deuteration of female mice with 25 per cent D_2O in the drinking water led to (1) a decrease in the number of breeding trials that eventuated in pregnancies that were carried close to term (TABLE 1, column 6), and (2) an increase in the percentage of nonviable litters (column 11). In general the incidence of near-term pregnancies decreased with increasing length of time on deuterated water. While the observed mean litter size decreased with increased time of exposure of the dams to deuterium, this situation is obscured by the finding that the percentage of pregnancies where no newborn were found ranged from 70 to 100 per cent when deuteration had preceded birth by at least 7 days. Comparable reduction of the number of visible pregnancies has been reported by Hughes and Calvin.[6]

Newborn mice that were deuterated *in utero* were maintained on 25 per cent D_2O in the drinking water for the first 100 days of life. Survival of these progeny is illustrated in FIGURE 4, which indicates that most of the deaths among the viable, deuterated newborn mice occurred during the first week.

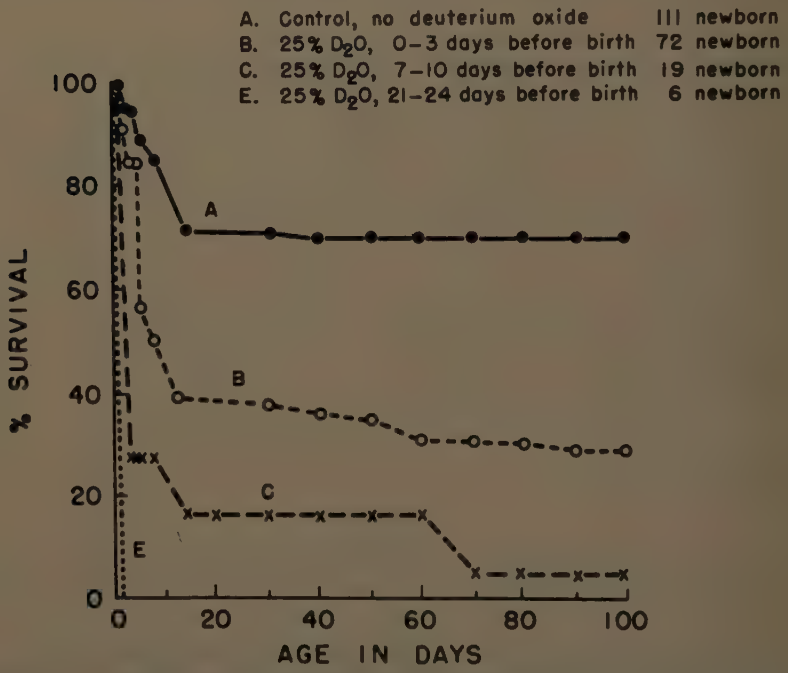

FIGURE 4. Survival curves for mice deuterated as fetuses and for the duration of life.

These results are consistent with those of Häggqvist,[7] who noted that with 5 mice the presence of 25 per cent D_2O in the drinking water led to stillbirths and maternal cannibalism of the newborn. Similar behavior of mice deuterated to 8 per cent was noted by Hansen and Wülfert.[8]

TABLE 2 presents the data obtained with those mice of the first experiment that had been deuterated for 7 weeks and then returned to ordinary water. In general, the incidence of pregnancy was somewhat low, and was correlated with the recency of the return to ordinary water intake. Mean litter size was similarly greatest for those mice in which the greatest amount of deuterium had been washed out of the body. A wholly nonviable litter occurred only in Group B in which deuterated drinking water was continued until parturition.

The evaluation of the effects of deuterium in the first series of experiments depended on criteria that are subject to a number of uncertainties such as undetected delivery of dead fetuses and the disappearance of viable newborn because of maternal cannibalism. The second series of experiments was designed to ascertain the actual numbers of viable and of dead fetuses during the last week of gestation in relation to several deuteration schedules. For this purpose, animals were sacrificed, as noted above, on the 18th day after mating was started, and the uterine contents were examined. Normal fetuses were counted, and the dead embryos were classified, in accordance with Bateman,[9] as early postimplantation deaths (ED) and late deaths (LD). The former consist of wartlike deciduomata representing embryos dying at or shortly after the time of implantation at 5 days; the latter are dead fetuses in various stages of later development and disintegration.

Table 2

Effects of Return of Pregnant Deuterated Mice to Ordinary Water

Group*	No. days on H_2O before delivery	No. mice	No. pregnant mice	Per cent pregnant	Number of litters: No newborn found	Number of litters: All newborn dead	Number of litters: At least one newborn alive	Per cent nonviable litters	Total No. observed newborn	Observed mean litter size	No. alive at 30 days: ♂	No. alive at 30 days: ♀
B	0	9	1	11.1	1	0	0	100	—	—	—	—
C	7	7	1	14.3	0	0	1	0	3	3.0	0	0
D	14	8	3	37.5	0	0	3	0	24	8.0	14	8
E	21	8	2	25.0	0	0	2	0	18	9.0	13	5

* Male mice were introduced into Group B, C, and D breeding cages that were supplied with 25 atom per cent D_2O in the drinking water, and they remained for 1 week. At 7 days mice drinking this level of deuterium oxide reach 95 per cent of the equilibrium value of deuteration of body fluids.[5]

Table 3

Results of Examination of Uteri of Near-term Mice after Varying Periods of Deuteration

Group	No. days on D_2O	No. mice	No. pregnant	Total fetuses (No.)	Total viable fetuses: No.	Total viable fetuses: %	Total nonviable fetuses: No.	Early fetal deaths (ED): No.	Early fetal deaths (ED): %	Late fetal deaths (LD): No.	Late fetal deaths (LD): %	Mean No. fetuses per pregn. ♀	Mean No. viable fetuses per pregn. ♀
A	0	30	13	147	136	92.5	11	10	6.8	1	0.7	11.3	10.5
B	4	10	4	42	40	95.2	2	2	4.8	0	0.0	10.5	10.0
C	11	10	5	59	54	91.5	5	2	3.4	3	5.1	11.8	10.8
D	18	10	6	60	41	68.3	19	17	28.3	2	3.3	10.0	6.8
E	25	10	3	35	24	68.6	11	8	22.9	3	8.6	11.7	8.0
F	32	10	5	47	28	59.6	19	16	34.0	3	6.4	9.4	5.6

The results of these examinations are given in TABLE 3, which shows, first of all, that pregnancy ensued under the conditions of the experiment in from 30 to 60 per cent of the mice in any group, and that the incidence of pregnancy was not correlated with the duration of deuteration. Second, the mean number of fetuses (including implantation sites) per pregnant female varied from 9.4 to 11.8, again apparently independently of the length of time on deuterated drinking water. Third, the mean number of apparently viable fetuses per pregnant female (as seen on the 13th to 18th day of gestation) varied from 10.0 to 10.8 in the control mice and in Groups B and C. This index, however, was reduced to values ranging from 5.6 to 8.0 in those mice that had been on D_2O for 18 or more days. In other words, 91.5 to 95.2 per cent of the fetuses in near-term mice that had been on 25 per cent D_2O in the drinking water for fewer than 12 days were alive, while only 59.6 to 68.6 per cent of the fetuses in mice similarly deuterated for a period longer than 18 days were viable.

These results reflect the increased incidence of early deaths when the onset of deuteration preceded or coincided with the beginning of mating (Groups D, E, and F). The percentage of deciduomata in these groups ranged from 23 to 34 per cent of the total number of detectable fetuses. On the other hand in the control group and in those where deuteration was started 7 or 14 days after the beginning of mating, the early deaths accounted for only 3 to 7 per cent of the total number of fetuses. The increased number of early deaths in Groups D, E, and F undoubtedly reflect the effects of deuterium at body fluid concentrations sufficiently high to be toxic to the early embryo, especially at the time of implantation on the fifth day.

TABLE 4

RESULTS OF EXAMINATION OF UTERI OF NEAR-TERM MICE AFTER 25 DAYS ON DIFFERENT LEVELS OF D_2O IN THE DRINKING WATER*

Group	Concn. D_2O	No. mice	No. pregnant	Total fetuses (No.)	Total viable fetuses		Total non-viable fetuses (No.)	Early fetal deaths (ED)		Late fetal deaths (LD)		Mean No. fetuses per pregn. ♀	Mean No. viable fetuses per pregn. ♀
					No.	%		No.	%	No.	%		
A	0	10	8	88	77	87.5	11	11	12.5	0	0.0	11.0	9.6
B	10	9	4	47	42	89.4	5	5	10.6	0	0.0	11.7	10.5
C	20	10	2	26	19	73.1	7	6	23.1	1	3.8	13.0	9.5
E†	25	10	3	35	24	68.6	11	8	22.9	3	8.6	11.7	8.0
D	30	10	3	24	0	0.0	24	19	79.2	5	20.8	8.0	0.0
Combined‡ controls	0	40	21	235	213	90.6	22	21	8.9	1	0.4	11.2	10.1

* D_2O administration started 7 days before beginning of 5-day mating period.

† Group E from TABLE 3.

‡ TABLES 3 and 4.

The effects of increasing the concentration of deuterium in the drinking water in the third experiment are given in TABLE 4, which includes data from TABLE 3 (Group E) and which also gives the combined data for the control mouse population from both experiments. The mean number of fetuses (dead and alive) per pregnant female appears to be unaffected by increasing concentrations of D_2O until a level of 30 per cent in the drinking water is reached. Of the population of fetuses observed, the total number of viable ones (as well as the mean number of viable fetuses per pregnant mouse) declined slowly with increasing deuterium concentration. Here, under the conditions of this experiment, a critical level seems to exist between 25 and 30 atom per cent deuterium in the drinking water since fetal viability ceased at the latter level. At the lower deuterium levels, fetal death for the most part occurred early, that is, soon after implantation, and while the percentage of early deaths increased with increasing D_2O concentration, an additional increment of mortality from late fetal deaths was present at higher concentrations.

Discussion

It is apparent from the results of the examinations of uterine contents that 25 per cent D_2O in the drinking water did not adversely affect the mean number of fetuses *implanted* even when deuteration preceded mating by 7 to 14 days. On the other hand, imbibition of this concentration of D_2O starting 0 to 14 days before mating and continuing through pregnancy (Groups D, E, and F, second experiment) did result in a decrease in the mean number of *viable* fetuses seen 3 to 7 days before term. This mean value dropped from 10.4 to 6.4 (approximately 35 per cent). Since the males, having had only an overnight exposure to D_2O, were essentially unaffected by the deuterium, this result would indicate that females deuterated to a level of 18 to 20 per cent in the body fluids did not suffer an impairment in the ability to become pregnant, that is, implantation of fetuses in the placental tissue. Such female mice, however, did demonstrate a reduced over-all reproductive potential because of the toxic effects of deuterium on roughly one third of the implanted fetuses early in development. Such deuteration also increased to a slight extent the incidence of deaths later in gestation (LD). No attempt was made in these studies to determine the number of preimplantation deaths, but the similarity of the total number of fetuses per pregnant female in all groups suggests that the preimplantation stages of development are not affected by deuterium in the concentrations used in these studies.

The first series of experiments established the increase in nonviability of the newborn with increasing duration of deuteration. This increase in nonviability became apparent when the mother was deuterated just a few days prior to delivery, and it was marked when preparturition deuteration lasted 7 to 10 days or longer. The extent to which this increase in nonviability is attributable to toxic effects late in embryological development or to behavior changes in the parturient female is not entirely distinguishable from our observations. While the percentage of viable fetuses seen in the uterine horns dropped when deuteration extended through the gestation period, many presumably parturient mice were found to lack newborn litters. In these cases, the newborn mice were undoubtedly eaten by the mother, either because they were dead or dying, or possibly also because the deuterium may have altered the thirst pattern of the dams so that they destroyed the newborn in an effort to satisfy the need for ordinary water.

It has been asserted that substitution of D_2O for a part of the drinking water has produced sterility in mice where *sterility* was specifically defined as "the inability to produce visible pregnancy."[6] We have felt, in discussing a complex array of events in female mice that includes cyclical mating behavior, fertilization of the ovum, implantation of the fetus, successful gestation and parturition, viability of the newborn, and postpartum behavior of the dam, that a more suitable term is *reproductive potential*. In this way, female mice cannot be called sterile in the face of unaffected ability to become pregnant although a toxic effect on the implanted fetuses may reduce the reproductive potential in this situation. Similarly, concepts involving sterility are not invoked when there is evidence that the number of young mice 2 weeks after birth may have been reduced because of maternal behavior rather than by interference with physiological processes involved in reproduction.

Adult female mice have been maintained on 25 per cent deuterium oxide in the drinking water for as long as 280 days without adverse effect on their body weight.[10] This level of deuterium in the drinking water leads to body fluid deuterium levels of about 18 to 20 per cent. The poor survival of mice born of deuterated mothers when comparable deuteration was continued in the newborn and young mice for 100 days may reflect the depressant action of deuterium on rapidly growing tissues.

The results with deuterium presented here may in general be ascribable to the deuterium isotope effect operating through the greater stability of deuterium bonding with carbon, although the detailed mechanism has not been elucidated at this time.[2,3] It is also possible that extensive replacement of hydrogen by deuterium in an organism is in part physiologically equivalent to dehydration because of the dilution of hydrogen by a more inert isotope.

Summary

Replacement of hydrogen with deuterium by the administration of 25 per cent D_2O in the drinking water resulted in certain alterations in the reproductive potential of female mice. These changes included an apparent decrease in the number of pregnancies carried close to term, an increase in the incidence of wholly nonviable litters, and a decrease in the survival of newborn mice. Examination of uterine contents late in pregnancy, however, revealed that neither the incidence of pregnancy nor the mean number of implanted embryos per pregnant female was correlated with the duration of deuteration. Reduction of the incidence of viable newborn mice in part reflected the increase in nonviability of fetuses examined *in utero* late in gestation, and in part may have been the result of maternal cannibalism. Reduction of fetal viability was greatest when the dams had been substantially deuterated at the time of implantation of the fetus, which occurs at about the fifth day after mating. When the administration of deuterium to female mice preceded mating by 1 week, the higher the level of deuteration, the lower was the total fetal viability. A critical level appeared to exist here between 25 and 30 atoms per cent deuterium in the drinking water, above which fetal viability ceased. The reduction of mean litter size and of viability of the newborn was at least partly reversible by restoring the dams to ordinary drinking water.

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CONCLUDING REMARKS ON THE CONFERENCE ON DEUTERIUM ISOTOPE EFFECTS IN CHEMISTRY AND BIOLOGY

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This publication may be, in my opinion, a landmark in the history of deuterium research. The discovery of deuterium by H. C. Urey, F. G. Brickwedde, and G. M. Murphy in 1932 was followed by a host of chemical and biological researches. The predictions of Urey and G. N. Lewis that deuterium should have properties significantly different from those of hydrogen in biological systems were instrumental in provoking a most varied series of studies on many different living organisms. Nevertheless the subject soon lost interest, and by 1940 very little research, with the exception of deuterium tracer studies, was being carried out in this area. The reasons are not hard to find. The extraordinary scarcity and expense of deuterium made research very difficult; the investigator was forced to expend more effort on the conservation of the heavy water than on the experiments themselves.

Deuterium is no longer a laboratory curiosity, but is rather a large-scale industrial chemical. Thanks to the efforts of the United States Atomic Energy Commission, almost unlimited amounts of heavy water are now available, and it is now possible to contemplate experimentation with deuterium on the large scale. The success of the United States Atomic Energy Commission in producing deuterium and other stable isotopes on the large scale has been decisive in the revival of interest in the whole subject of stable isotope effects.

This monograph is rather unusual in the range of subject matter included. The contributors have ranged from chemical physicists and physical organic chemists on the one hand to virologists and cytologists on the other. The subjects have ranged from the application of deuterium to the elucidation of the mechanisms of organic reactions, to the effects of deuterium on algae, bacteria, and invertebrates; from the effects of deuterium on protein configuration, to effects on virus and tumor growth. The physiological effects of deuterium on mammals and on reproductive potential in mammals have both been among the subjects considered in these pages. However, although the range of subjects has been so great, it is difficult to avoid the conclusion that these represent only a small fraction of those that eventually will be studied.

The discovery that algae and some other microorganisms can be mass cultured in 99.8 per cent D_2O may have interesting repercussions. The way is now open to the biosynthesis of fully deuterated compounds and, as a matter of fact, compounds in which the stable nuclei are replaced not only by H^2, but by C^{13}, N^{15}, O^{18}, and S^{34}. Organisms and compounds in which the stable nuclei are arranged in different ways by combination and permutation may be valuable in research. Such isotopically altered organisms and compounds should be useful in nuclear magnetic resonance studies and electron spin resonance studies. Fully deuterated compounds should be valuable for neutron diffraction crystal structure studies. Fully deuterated compounds, because of altered reaction rates, may be metabolized at different rates and thus provide still another way to exercise control over cellular reactions. Biosynthesis is a uniquely useful method for securing fully deuterated compounds, since the synthesis of such substances as proteins, enzymes, and nucleic acids are beyond the present capacities of the organic chemist. These are readily provided by living organisms. Possible applications of deuterated organisms and compounds have received only a little attention in this publication. It would not be too surprising to find them the subject matter of a future one. Interest in the chemical and biological effects of deuterium and other stable isotopes will surely be stimulated by the publication of this monograph.

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